Endoplasmic Reticulum (ER)-stress signalling in the alveolar epithelium

Inaugural Dissertation
submitted to the
Faculty of Medicine
in partial fulfilment of the requirements
for the Ph.D. degree
of the Faculties of Veterinary Medicine and Medicine
of the Justus Liebig University Giessen

by

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from

Lahn-Giessen

Giessen 2012

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Date of Doctoral Defence: 6. November 2012

CONTENT

Table of contents

List of figures

List of tables

Register of abbreviations

++	homozygous for a gene	AECII	Alveolar epithelial cells type II
+-	heterozygous for a gene	Amp	Ampicillin
++/++	homozygous for respective transgene/ and transactivator (rtTA)	APS	Ammonium persulphate
		aRNA	antisense RNA
+-/+-	heterozygous for respective transgene/ and transactivator (rtTA)	ATF4	activating transcription factor 4
		Atf4	gene for ATF4
A	Adenine		

ATF6	activating transcription factor 6 (here usually referring to the cleaved form of ATF6)
Atf6	gene for ATF6
ATP	Adenosintriphosphate
BAL	broncho-alveolar lavage
BLEO	bleomycin
bp	base pairs
BSA	bovine serum albumin
C	Cytosine
°C	degree Celsius
CHOP	C/EBP Homologous Protein
Chop	gene for CHOP
cm	centimeter
CRL	Charles River Laboratories (Kisslegg, Germany)
CT	cycle of threshold
dCT	delta CT
ddCT	delta delta CT
dd-water	double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxycycline
dNTP	Deoxy ribonucleotide triphosphate
ECL	enhanced chemo-luminescence
ECM	extracellular matrix
E. coli	*Escherichia coli*
EDTA	Ethylendiamin-tetra-acetic acid
	Ethylendinitrilo-N,N,N',N',-tetra-acetate
EMBLEM	Transgenic animal facility of EMBL (Heidelberg)
ER	Endoplasmic Reticulum
et al.	*et alii* (and others)
ETBr	Ethidiumbromid
Falcon	centrifugation tube
FCS	Fetal Calf Serum
FVB/N	an inbred strain of white mice
g	gram
G	Guanine
h	hour(s)
H&E	Haematoxylin & Eosin
HEPES	N-2-Hydroxy-ethylpiperazin-N-ethansulfonate
HRP	horseradish peroxidase
IHC	immuno histochemistry
IPF	Idiopathic Pulmonary Fibrosis
kan	Kanamycin
kb	kilo base pairs
kDa	kilo Dalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
l	litre
LB medium	Luria Bertani-complete medium
lfc	log fold change
m	milli
M	molar
M	Marker
MEL188	Human SK MEL-188 Melanoma Cells

min	minute
miRNA	micro RNA
MMP	matrix metallo-proteinase
mRNA	messenger RNA
MW	molecular weight
µ	micro
n	nano
NCBI	National Center for Biotechnology Information
OD	optical density
ON	over night
PAGE	Polyacrylamide Gel Electrophoresis
p90-ATF6	full length activating transcription factor 6
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PERK	Pancreatic eIF2-alpha kinase
PMN	polymorph nuclear cell
PTP	permeability transition pore
PVDF	polyvinylidene fluoride
qPCR	quantitative PCR
rcf	relative centrifugal force
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT	reverse transcription
SDS	sodium dodecyl-sulphate
sec	second(s)
snRNA	small nuclear RNA
SP	surfactant protein
Taq	DNA-Polymerase from *T. aquaticus*
TAE	TRIS / Acetic acid / EDTA buffer
TG	Thapsigargin
TRIS	Tris(hydroxyl methyl)-amino methane
tRNA	transfer RNA
Tween 20	Polyoxyethylen(20)-sorbitanmonolaurate
U	unit(s)
UPR	unfolded protein response
UV	ultra violet
V	Volt
WB	Western blot
WT	wild type
XBP1	X-box-binding protein 1 (here usually referring to the spliced form)
www	world wide web
Xbp1	gene for XBP1 (here usually referring to the spliced form)

SUMMARY

Endoplasmic Reticulum (ER)-stress, the accumulation of misfolded proteins in the ER, is assumed to be the cause of several diseases. Recently, enough evidence accumulated to state that it also plays an important role in idiopathic pulmonary fibrosis (IPF). The presence of a maladaptive ER-stress in the alveolar epithelial type II cells (AECII) of patients' lungs has been demonstrated and the ability of this mechanism to cause or enhance a fibrotic phenotype has been shown. The hypothesis that AECII apoptosis induced by ER-stress or alternative mechanisms is a main trigger of this disastrous disease is therefore widely accepted. Nevertheless it was a requirement to characterize the diverse pathways of ER-stress response (the unfolded protein response, UPR) in the alveolar epithelium in detail.

My studies focus on, but are not limited to, the effects of the UPR- transcription factors Atf4, Atf6 and Xbp1 *in vitro* in the AECII-derived murine cell line MLE12 and *in vivo* with conditional over-expression in transgenic mouse lungs of *de novo* generated lines. A broad analysis of the transgene-effect *in vitro* was assessed, based on microarray analysis of transfected cells, including miRNA analysis and 400K-Exonarrays, which provide first evidence that regulation of miRNAs and alternative splicing events are components of the UPR. Induction of cell death by the UPR-transcription factors was of special interest, because it is a proposed mechanism in the pathogenesis of IPF. However, no increased cell death or strong induction of the pro-apoptotic mediator Chop (gene for the C/EBP Homologous Protein; CHOP) was observed, the protective effects seemed to dominate in the system. Atf4, Atf6 and Xbp1 are not sufficient to mimic or generate a maladaptive ER-stress-situation in these cells. In experiments with drug (Thapsigargin, Brefeldin A) induced ER-stress the induction of Chop was one of the earliest effects and occurred simultaneously with the induction of Atf4 and Xbp1(s). Microarrays, addressing the very early effects of Thapsigargin (TG) treatment, could reveal an even earlier transcriptional regulation. However, it could not be determined which factors make the difference between a "good" and a maladaptive ER-stress. Other potential impact of ER-Stress on IPF resulting from the expression data was discussed.

Transgenic mice for inducible over-expression of Atf4, Atf6 and Xbp1 were generated. The investigated lines did not develop any phenotype after transgene induction. This would fit to the *in vitro* finding, that the transgenes do not cause

AECII death. However, the extend of the transgene induction *in vivo* has not yet been completely assessed.

Zusammenfassung

Endoplasmatischer Reticulum (ER)-Stress, die Akkumulation fehlgefalteter Proteine im ER, wird als Ursache einer ganzen Reihe von Krankheiten angesehen. In letzter Zeit haben sich genug Belege angesammelt, dass er auch eine wichtige Rolle in der idiopathischen pulmonalen Fibrose (IPF) spielt. Die Präsenz eines maladaptiven ER-Stress in den alveolären Typ II Zellen (AECII) von Patientenlungen und die Fähigkeit dieses Mechanismus einen fibrotischen Phänotyp zu erzeugen oder zu verstärken, wurden gezeigt. Die Hypothese, dass durch ER-Stress oder alternative Mechanismen induzierte AECII Apoptose, ein Hauptfaktor bei dieser tödlichen Krankheit ist, wird daher weitgehend anerkannt. Dennoch war es erforderlich, die diversen Wege der ER-Stress Antwort (UPR) in AECII detailliert zu charakterisieren.

Meine Forschungen waren auf die Effekte der UPR-Transkriptionsfaktoren Atf4, Atf6 und Xbp1 *in vitro* in der murinen Zelllinie MLE12 fokussiert aber nicht darauf begrenzt. Sie umfassten *in vivo* die konditionale Überexpression dieser Faktoren in den Lungen *de novo* generierter transgener Mauslinien. Eine breit angelegte Analyse der Transgeneffekte *in vitro*, wurde basierend auf Microarray-Analysen transfizierter Zellen, inklusive 400K Exonarrays und miRNA-Arrays, vorgenommen, die erste Belege liefern, dass miRNA Regulation und alternatives Spleißen Teile der UPR sind. Induktion von Zelltod durch diese UPR-Transkriptionsfaktoren wird im engen Zusammenhang mit der Pathogenese von IPF diskutiert. Daher wurde Zelltod durch UPR-Transkriptionsfaktoren näher untersucht. Allerdings wurde kein verstärkter Zelltod oder eine starke Induktion des proapoptotischen Mediators CHOP beobachtet, die schützenden Effekte schienen in diesem System zu dominieren. Atf4, Atf6 und Xbp1 sind nicht hinreichend, um eine maladaptive ER-Stresssituation in diesen Zellen zu simulieren oder zu erzeugen. In Experimenten mit durch Chemikalien (Brefeldin A, Thapsigargin) erzeugtem ER-Stress war die Induktion von CHOP eines der frühesten Ereignisse und trat simultan mit der Induktion von Atf4 und Xbp1(s) auf. Microarrays, die auf die sehr frühen Effekte von Thapsigarginbehandlung abzielten, konnten noch frühere, transkriptionale Reaktionen zeigen. Allerdings konnte nicht bestimmt werden, welche Faktoren den Unterschied zwischen einem „guten“ und einem maladaptiven ER-Stress ausmachen. Andere potentielle Einflüsse von ER-Stress auf IPF, die sich aus den Expressionsdaten ergaben, wurden diskutiert. Transgene Mäuse für die induzierbare Überexpression von Atf4, Atf6 und Xbp1 wurden generiert. Die untersuchten Linien entwickelten keinerlei

Phänotyp als Reaktion auf die Transgeninduktion. Das würde zu den *in vitro* Befunden passen, dass die Transgene keinen AECII-Zelltod erzeugen. Allerdings wurde das Ausmaß der Transgeninduktion *in vivo* noch nicht vollständig untersucht.

1. INTRODUCTION

1.1 Idiopathic pulmonary fibrosis (IPF)

Idiopathic pulmonary fibrosis is a unique type of chronic fibrosing lung disease of unknown aetiology. IPF is characterized by epithelial injury and activation, the formation of distinct sub-epithelial fibroblast / myofibroblast foci and excessive ECM (extracellular matrix) accumulation. These progressive and irreversible changes in the lung architecture result in progressive respiratory insufficiency and a fatal outcome in a relatively short period of time (Pardo, Selman 2002), usually 2-4 years after diagnosis (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias 2002). Histological, temporal and spatial heterogeneity exists, resulting in still regular appearing septae directly adjacent to disease-defining areas such as fibroblastic foci, honeycomb changes with dense fibrosis and hyperplastic epithelium. Some authors carefully propose that IPF and another form of idiopathic interstitial pneumonia, the so called Non-specific Interstitial Pneumonia (NSIP), represent different forms of manifestation of one and the same molecular trigger. Evidence for such reasoning stems from the fact that histologic patterns of IPF as well as NSIP can occur simultaneously in the same lung lobe and in families with SP (surfactant protein) -C mutations also cases of NSIP occurred (reviewed by Thannickal *et al*. 2004).

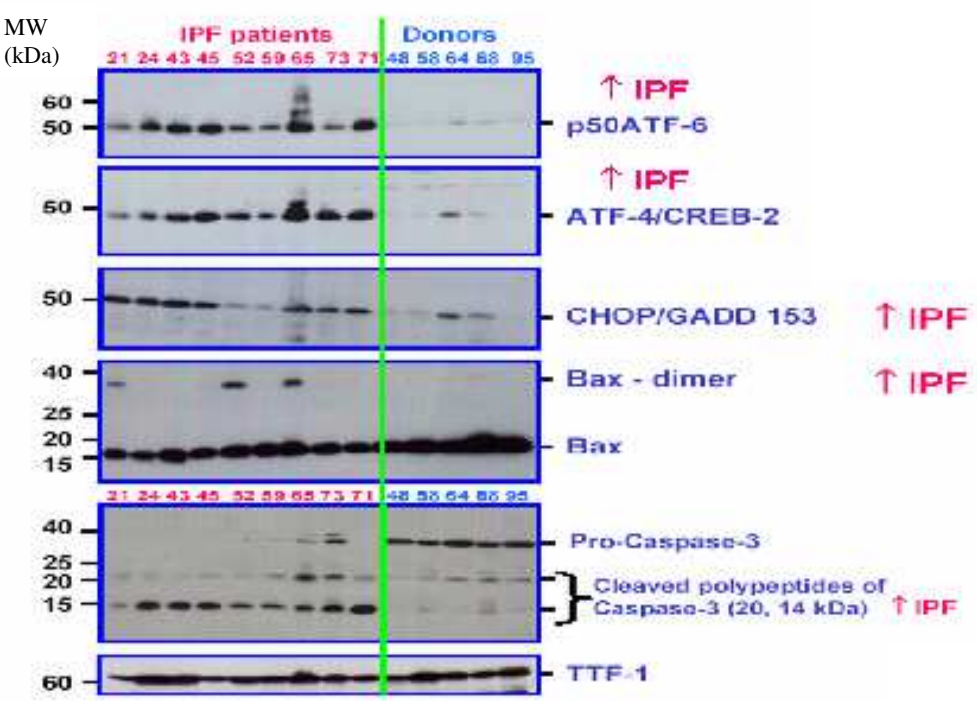
Previous research has largely focused on putative inflammatory mechanisms underlying the initiation of the fibrotic response, but recent evidence strongly suggests that chronic epithelial injury is the key underlying pathogenic event (Pardo, Selman 2002).

The inflammatory hypothesis suggested that IPF results from chronic inflammation in response to a yet undefined stimulus. If left untreated, it would lead to progressive lung injury and ultimately fibrosis (American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias 2002). This view was based on the obvious signs of inflammation on histological, broncho alveolar lavage (BAL) (neutrophilic alveolitis) and radiographic (enlarged lymph nodes, modest ground glass opacities) findings. However, anti-inflammatory therapies largely failed to gain any meaningful clinical effect. This finding is questioning the concept that inflammatory events play a causative role may substantially contribute to the

progression of the disease (reviewed by Günther *et al*. 2006; Verma, Slutsky 2007; Markart *et al*. 2006; Selman *et al*. 2001, Mahavadi 2009).
However, it must be taken into account that the role of inflammation in IPF is not zero (Montaldo *et al*. 2002) and that also ER-stress (later on introduced as a factor in the pathogenesis of IPF) can induce inflammatory responses, mainly by activating NFκB-signalling (reviewed by Zhang, Kaufman 2008).
When inflammation became more and more unlikely as trigger of the disease, chronic epithelial injury was still often regarded as an epiphenomenon (example: Chua *et al*. 2005), while other authors valued the role of epithelial injury much stronger (examples: Geiser 2003; Myers, Selman 2004; Myers, Katzenstein 1988). Others adopted the view, that epithelial injury can play a causative role in IPF but still highlighted the ongoing inflammatory events (Gharaee-Kermani, Phan 2005). Some other authors focused on the imbalance of anti-fibrotic factors [MMPs (matrix metallo-proteinases), CD44] and pro-fibrotic factors (tissue inhibitor of MMPs, TGFß and ECM components) without asking for a general trigger mechanism (Cook *et al*. 2002). Indeed, scarring of the lung is a multistep and multifactor phenomenon (review by Razzaque, Taguchi 2003). It was also pointed out that significant biochemical and biophysical alterations occur predominantly in IPF and may contribute to the disturbances of alveolar surface tension stability and gas exchange in this disease (Schmidt *et al*. 2002).
External factors have also been indicated to contribute to the development of IPF (Steele *et al*. 2005). The strongest evidence exists for exposure to cigarette smoke and occupational exposure to metal dust (reviewed by Selman, Pardo 2006).
Viral infection has also been suggested to play a role in IPF and replicating virus in the presence of an exogenous injury may indeed promote the development of pulmonary fibrosis (Lok *et al*. 2002). Diverse viruses were found to be associated with IPF, namely EBV (Tsukamoto *et al*. 2000), for which replication in IPF patients AECII was also shown (Egan *et al*. 1995), cytomegalovirus and human herpes viruses (HHV)–7 and HHV-8 (Tang *et al*. 2003). It has however to be stated that these studies do have limitations. In a more recent publication, there was no convincing evidence for a distinct pathogene signature in IPF (Wootton *et al.* 2011). Also Acid-aspiration (by gastroesophageal reflux)–induced epithelial injury may contribute to the development of IPF.
However, external stimuli are unlikely to cause IPF without accompanying genetic alterations. Firstly, telomerase mutations and subsequent shortening of telomeres were found to be associated with a subset of familial cases of IPF. This leads to apoptosis and cell cycle arrest (Armanios *et al*. 2007). Shortening of telomere

length can also occur independently of those mutations in IPF. TGF-ß can suppress human telomerase activity (Li *et al*. 2006) and oxidative stress accelerates telomere loss (von Zglinicki 2002). Secondly, mutations of SP-C have been demonstrated to be associated with familial forms of IPF (Nogee *et al*. 2001; Cameron *et al*. 2005). It has been shown that the mutated SP-C cannot fold correctly and induces apoptosis of the alveolar type II cell (AECII) (Mulugeta *et al*. 2007). Over-expression of mutated SP-C *in vivo* results in an exaggerated response to profibrotic stimuli (Lawson *et al*. 2011). As SP-C is exclusively produced by AECII, the mechanism clearly starts by affecting this alveolar epithelial cell type. Mutations in Surfactant protein A2 have also been associated with IPF (Wang *et al*. 2009) and could trigger the disease by similar mechanisms. Furthermore there is evidence, that targeted AECII injury is sufficient to induce a fibrotic response (Sisson *et al*. 2010) even though such drastic consequences do not occur in models where alveolar septal cell apoptosis can be outbalanced by increased cell turnover (Fehrenbach *et al*. 2007).
In sporadic cases of IPF SP-C mutations are rare (Markart *et al*. 2007; Lawson *et al*. 2004) but a similar injurious mechanism was proposed. Markers for an ongoing UPR and epithelial apoptosis are up-regulated in IPF versus donor patients lung homogenates and these markers could be localized to the hyperplastic AECII of those lungs by immunostaining (see *figure 1*) (Korfei *et al*. 2008). Apoptotic AECII death also occurs in normal alveoli of IPF / UIP, although on a lower level (Barbas-Filho *et al*. 2001).

As underlying reason, defective processing of SP-C and SP-B and accumulation of their pro-forms, viral infection and the impact of reactive oxygen species (ROS) have been implicated (Lawson *et al*. 2008). IPF proteome analysis revealed evidence for chronic epithelial ER-, oxidative - and DNA (Deoxyribonucleic acid) damage stress, resulting in epithelial instability and cell death, paralleled and confirmed by signatures of enhanced infection susceptibility and deterioration of alveolar structure (Korfei *et al*. 2011).



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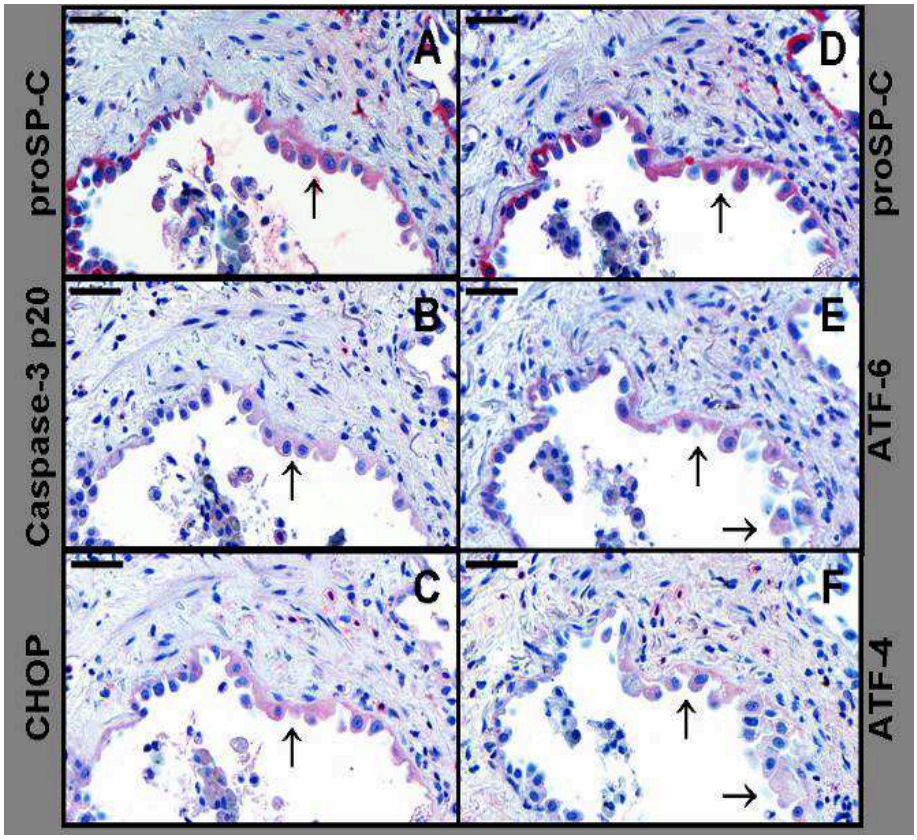

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***Figure 1*: Maladaptive endoplasmic reticulum stress in sporadic IPF (continued).**

***Figure 1*: Maladaptive endoplasmic reticulum stress in sporadic IPF.** 1: The UPR-transcription factors (cleaved) ATF6 and ATF4 as well as the pro-apoptotic mediator CHOP and cleaved caspase-3 were more abundant in IPF-patients versus donor lung homogenates. In some samples dimerization of Bax could also be demonstrated. Numbers above the columns are patient numbers. 2: Caspase 3 cleavage (B) and induction of CHOP (C), ATF6 (E) and ATF4 (F) were localized to the hyperplastic AECII of IPF patients histologic slides as confirmed by staining of parallel sections with the Type II cell marker pro-SP-C (A+D). Bar=50µm. (Korfei *et al*. 2008), modified by the author.

The concept that AECII-apoptosis represents an early step in the IPF pathogenesis immediately suggests inhibition of this mechanism as a therapy. Indeed caspase inhibitors reduce fibrosis in the bleomycin (BLEO) model of lung fibrosis and have been proposed as a therapeutic approach in pulmonary fibrosis (Kuwano *et al*. 2001). They can reduce the number of apoptotic cells, the pathological grade of lung inflammation and fibrosis, and the hydroxyproline content in lung tissues in this model (Kuwano *et al*. 2001; Wang *et al*. 2000). The anti-fibrotic agent captopril also acts as an anti-apoptotic agent. This suggests that inhibitory actions of captopril on pulmonary fibrosis may be related to prevention of lung epithelial cell apoptosis (Uhal *et al*. 1998a). On the other hand, extent or profusion of fibroblastic foci in lung biopsies is strongly correlated with increased mortality in patients with IPF. There is evidence that myofibroblasts in IPF acquire resistance to apoptosis (reviewed by Thannickal, Horowitz 2006). A caspase inhibitor treatment in IPF might therefore actually cause a deterioration of fibrotic remodelling. It might therefore be an advantage to study the trigger mechanism for AECII apoptosis in IPF first.
N-acetylcysteine versus placebo for patients under treatment with prednisone plus azathioprine significantly slowed the deterioration of pulmonary function and is therefore widely used as off-label treatment. However, the anti-fibrotic drug pirfenidone has been recently approved for the treatment of adult patients with mild to moderate idiopathic pulmonary fibrosis in the European Union (reviewed by Costabel, Bonella 2011).

1.2 Endoplasmic reticulum (ER)-stress

1.2.1 General concept

A main function of the ER is the correct folding of ER-localized proteins, namely secretory and transmembrane proteins. ER-stress is the accumulation of unfolded

or misfolded proteins in the endoplasmic reticulum. This problem generally occurs, when the folding machinery cannot match the protein load (reviewed by Harding *et al*. 2002). High expression of large, heavily modified proteins, expression of mutant, folding-incompetent proteins and such processes which induce higher expression of secretory proteins, like cell differentiation of B-cells to plasma cells and viral infection can lead to ER-stress (reviewed by Schröder, Kaufman 2005). The ER also synthesizes sterols and lipids. Perturbations in lipid metabolism and transport can also lead to an ER-stress response (Ron, Oyadomari 2004; Zhang, Kaufman 2003; Shechtman *et al*. 2011).

1.2.2 The unfolded protein response (UPR)

When ER-stress occurs, the cell responds by the UPR. The UPR is a set of reactions, consisting of a general translational attenuation, induction of the components of the ER-folding machinery and the ER-associated degradation machinery (ERAD) (see *figure 2*).
When the ER-stress is to strong or prolonged (Araki *et al*. 2003; Bernales *et al*. 2006) the UPR drives the cells into apoptosis[1]. This process is called “maladaptive ER-stress”. Such “maladaptive ER-stress” may represent an adaptive response. For example, UPR mediated apoptosis of ß-cells was identified as a mechanism to return the maternal rat pancreas back to a non-pregnant state after delivery (Bromati *et al*. 2011). On the other hand, maladaptive ER-stress clearly represents an important event in the pathomechanism of several chronic diseases including IPF diabetes and neurodegenerative disease (also see 1.2.4).
The current model which describes how the UPR is mediated is rather simple. Three different receptors in the ER-membrane are activated upon ER-stress. The activation of the receptors involves the dissociation of the chaperone Grp78 (BIP) from these receptors. Originally the idea was that Grp78 is recruited to unfolded proteins and thereby removed from the receptors driven by the concentration gradient. However, more recent evidence indicates that the removal of BIP is not a passive process (Shen *et al*. 2005), that reduced levels of BIP are not sufficient to induce the UPR (Gewandter *et al*. 2009) and that inability of mutant Ire1 to bind BIP does not result in a constitutive activation (Oikawa *et al*. 2007). In conclusion, the activation is a more complex process (reviewed by Hetz, Glimcher 2009). The exact mechanism of activation of the ER-stress-receptors remains unknown. Once activated, the receptor PERK dimerizes and catalyzes the phosphorylation of the

[1] Please see the Discussion for critical remarks on this topic

eukaryotic initiation factor 2 alpha (eIF2α). The result is a general translational block on the one hand and paradoxically the up-regulation of the transcription factor ATF4 on the other hand.

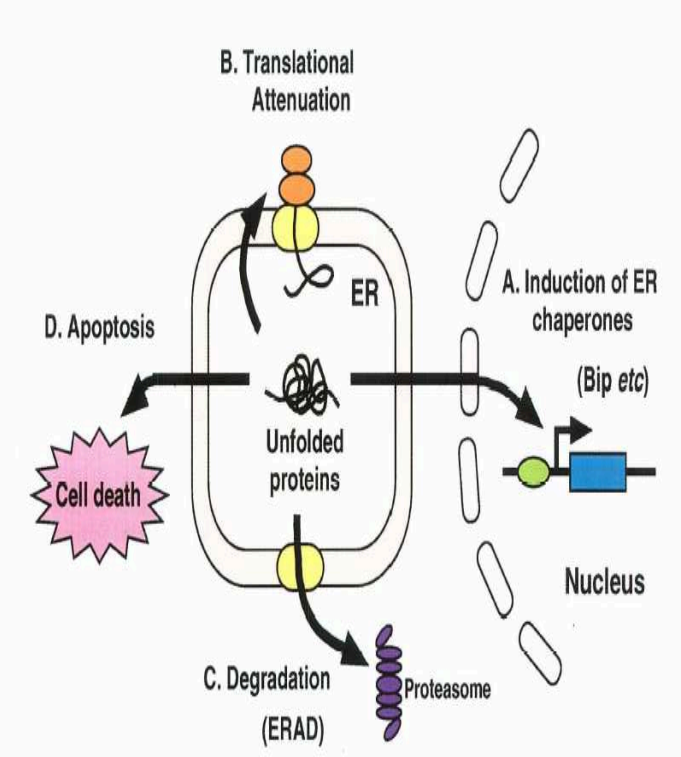


***Figure 2*: Principles of the unfolded protein response (UPR).** The accumulation of unfolded proteins is leading to the UPR, a combination of different responses. A) The induction of ER-chaperones like BIP and GRP94 to enhance the folding capacities of the ER, B) the up-regulation of the ER-associated protein degradation machinery (ERAD) to enhance degradation the of accumulated misfolded proteins by the proteasome and C) a general inhibition of protein synthesis. D) Induction of apoptosis can also be a part of the UPR. This is commonly addressed as "maladaptive ER-stress" and is regarded as a reaction against prolonged ER-stress or ER-stress above a certain threshold (Araki *et al*. 2003).

The receptor IRE1 also dimerizes, when activated and leads to the splicing of a 26bp intron from the Xbp1 mRNA (Lee *et al*. 2002; Back *et al*. 2006; Zhou *et al*. 2006). The splicing process equals tRNA splicing not nuclear mRNA-splicing via the spliceosome. Both the spliced form and the unspliced form of the Xbp1 protein are expressed. Spliced Xbp1 (Xbp1(s)) is an active transcription factor. Unspliced Xbp1 can dimerize with the Xbp1(s) and leads to its nuclear export (via an export sequence). Therefore it counteracts the Xbp1(s)-function (Yoshida *et al*. 2006).
When activated, the receptor p90-ATF6 is cleaved of the ER-membrane by S1P and S2P (Ye *et al*. 2000). The released 50 kDa (kilo Dalton) fragment (p50ATF6,

here usually just referred to as ATF6) can also act as a transcription factor (Haze *et al*. 1999; Shen, Prywes 2005).
Together ATF4, ATF6 and XBP1 are supposed to be responsible for most of the UPR-signalling including the rescue pathways and, paradoxically, the intrinsic pro-apoptotic pathways as well (reviewed by Bernales *et al*. 2006).

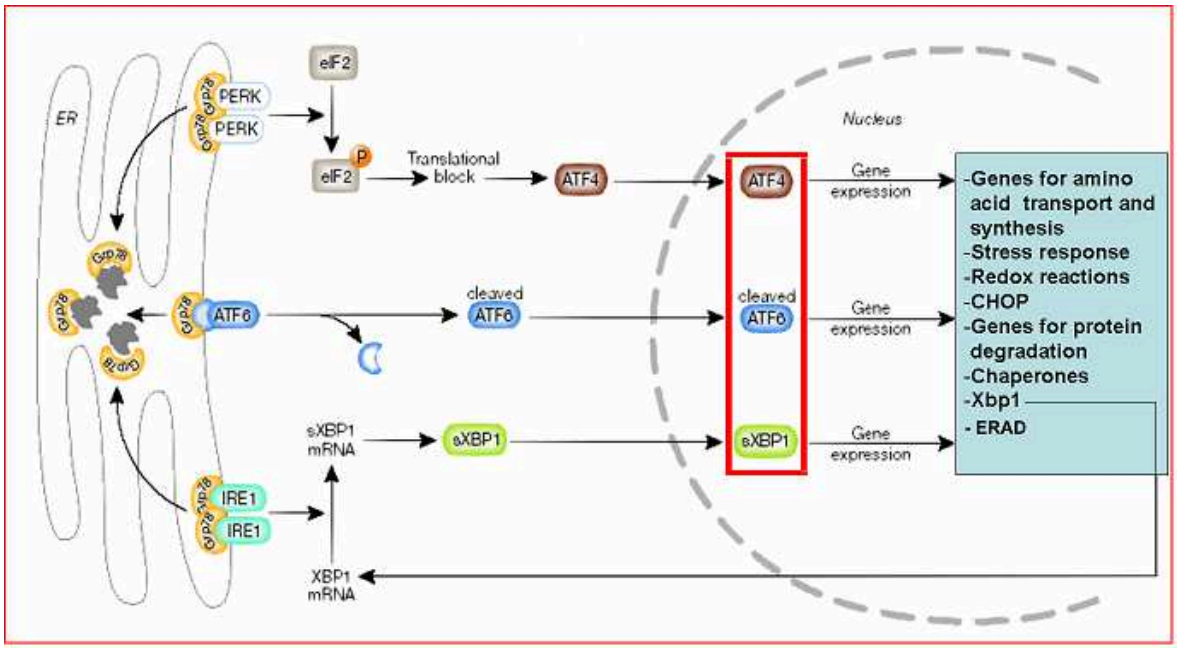


***Figure 3*: Pathways of UPR signalling.** The three main signalling pathways of the UPR are initiated by a process involving the dissociation of the chaperone BIP from the ER-membrane localized receptors PERK, p90-ATF6 and IRE1. PERK dimerizes when activated and then catalyzes the phosphorylation of eIF2 alpha which is responsible for a general translational block and induction of the transcription factor ATF4. Activated ATF6 is cleaved to its 50 kDa form which also acts as a transcription factor. IRE1 activation catalyzes the splicing of a 26bp intron from Xbp1 mRNA, leading to expression of the active transcription factor XBP1(s). Together the transcription factors ATF4, p50ATF6 and XBP1(s) (highlighted by a red frame) are then responsible for most of the downstream target expression of the UPR. They were reported to be responsible for the activation of the stress-resolving pathways and, paradoxically, also the pro-apoptotic pathways by up-regulation of CHOP (Szegezdi *et al*. 2006, modified).

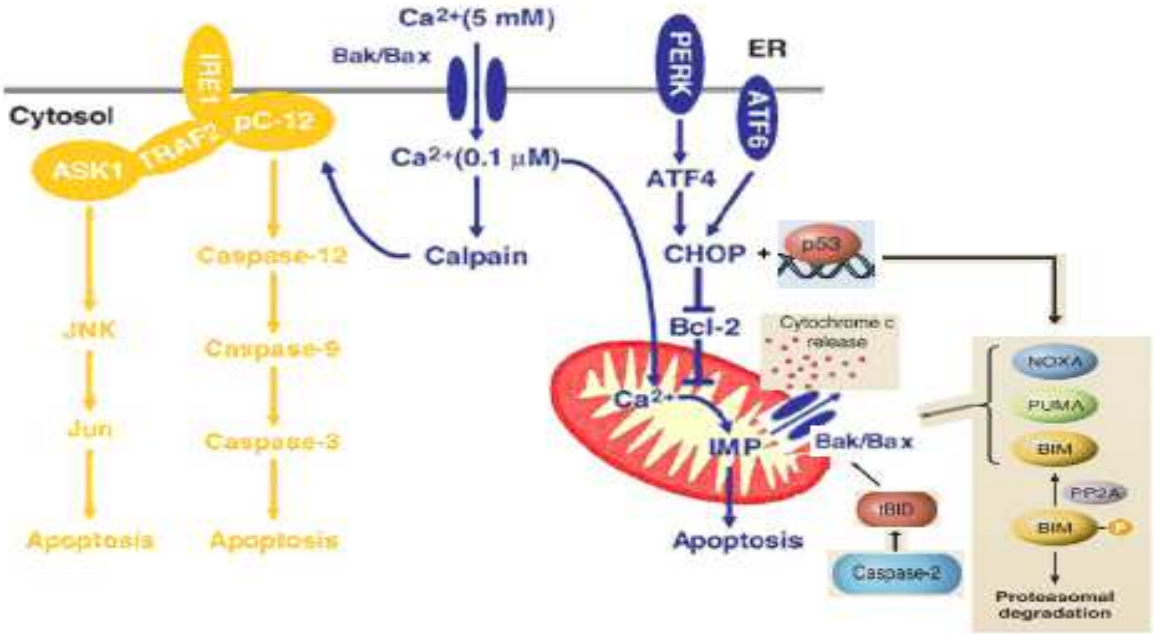
The activation of NFκB by ER-stress and its downstream signalling events represent a separate mechanism for the cell to react to ER-stress and is called ER-overload response (EOR) (Knorre 2001; Kuang *et al*. 2005; Rutishauser, Spiess 2002).
The UPR does not follow a uniform or simple, general scheme. Xbp1 for example binds different sets of gene promoters pending on the cell type and the induction

of ER-stress. It furthermore binds to target-promoters which participate in processes which were described as unexpected, including regulation of gene expression and chromosomal architecture, cell growth and differentiation, RNA processing and export, signal transduction, ubiquitin-associated processes, ion channels, transporters, and proton pumps (Acosta-Alvear *et al*. 2007).
ATF4, ATF6 and XBP1 belong to the group of basic leucine zipper (bZIP) -transcription factors. Their bZIP domain allows homo- or heterodimerization, also with numerous other bZIP-transcription factors. The diverse interactions allow condition-specific binding to different target sequence and thereby a very flexible and complicated downstream signalling (as summarized by Schröder, Kaufman 2005; Zhang, Kaufman 2004).

1.2.3 ER-stress and apoptosis

ER-stress can lead to apoptosis of the affected cell through different mechanisms (see *figure 4*). Following the extrinsic pathway, increased phosphorylation of tumor necrosis factor receptor-associated factor 2 (TRAF2) induced by c-Jun N-terminal inhibitory kinase (JIK) and its interaction with IRE1α and apoptosis signal-regulating kinase 1 (ASK1) results in activation of ASK1 and c-Jun amino terminal kinase (JNK), which phosphorylates and thereby inactivates BCL2 and thereby induces cell death. Furthermore the release of pro-caspase-12 from TRAF2 (when TRAF2 is sequestered to IRE1α) is considered to be necessary for pro-caspase-12 activation in humans (caspase 4 in mice).
With regard to the intrinsic pathway, insertion of oligomeric Bak and Bax into the ER membrane causes efflux of Ca^{2+} from the ER. The increase in the cytosolic Ca^{2+} concentration activates calpain, which cleaves and activates ER-localized pro-caspase-12. Caspase-12 activation is specific for and necessary in ER-stress mediated apoptosis (Nakagawa *et al*. 2000). Activated caspase-12 cleaves pro-caspase-9, and caspase-9 activates the executioner caspase, pro-caspase-3. Ca^{2+} released from the ER is rapidly taken up by mitochondria, which may lead to collapse of the inner membrane potential. Ultimately, Ca^{2+} influx into mitochondria opens the permeability transition pore (PTP), which is formed from a complex of the multi protein voltage-dependent anion channel, the adenine nucleotide translocase, and cyclophilin-D. Cytochrome c is then released through the PTP into the cytoplasm, where the apoptosome is formed and pro-caspase-3 is activated. In addition, the PTP recruits Bax to the outer mitochondrial membrane. Over-expression of an anti-apoptotic Bcl-2 family member, e.g., Bcl-XL, blocked

depolarization of the inner mitochondrial membrane in response to ER-stress. ATF4, ATF6 and XBP1 induce the expression of the pro-apoptotic bZIP – transcription factor CHOP[2]. CHOP represses transcription of anti-apoptotic Bcl-2 and thus shifts the balance between pro- and anti-apoptotic Bcl-2 family members toward the pro-apoptotic family members; chop–/– cells are partially resistant to apoptosis, which is consistent with this model (Schröder, Kaufman 2005; Oakes *et al*. 2006; Kaufman 2002). In cooperation with Elk1, CHOP is also up-regulating transcription of death receptor 5 (DR5) (Oh *et al*. 2010; Ohtsuki T *et al*. 2009).
It is noteworthy, that according to this model, CHOP is affecting the other apoptosis inducing pathways in the sense of an enhancement, but itself is mainly regulated by expression of the active ATF4, ATF6 and Xbp1. Indeed the role of these factors in Chop induction has been reported in different systems (Allagnat *et al*. 2010; Ma *et al*. 2002) and the over-expression of Chop was sufficient to induce apoptosis or cell cycle arrest in some models (Zinszner *et al*. 1998; Friedman 1996; Matsumoto *et al*. 1996).



F*igure 4*: ER-stress and apoptosis. ER-stress mediated apoptosis pathways can be described as an intrinsic pathway (blue) and an extrinsic pathway (yellow). The intrinsic pathway mainly works via induction of CHOP, which was described to be induced by ATF4, ATF6 and (not indicated here) XBP1(s). The extrinsic pathway is initiated via pro-apoptotic signalling complexes involving the receptor IRE1 (which is also responsible for Xbp1-splicing). Pro-caspase-12 (pC-12) activation and subsequent apoptosis via the effector caspase-3 requires activation of both, the extrinsic and the intrinsic pathway. IMP=inner mitochondrial membrane potential (Schröder, Kaufman 2005 modified according to Hetz et al. 2011).

[2] Please see the Discussion for critical remarks on this topic

1.2.4 ER-stress and disease

ER-stress can play very diverse roles. For example, UPR-components gained importance in the field of neurosciences. Restraint stress and amphetamine administration robustly induce ATF2, ATF3, and ATF4 in the nucleus accumbens and dorsal striatum and increase (ATF2) or repress emotional reactivity (Green *et al*. 2008). Lack of eIF2α-phosphorylation increases synaptic plasticity and is leading to increased long term memory in mice, without altering brain morphology (Costa-Mattioli *et al*. 2007; Costa-Mattioli 2008). The most intriguing feature of ER-stress might be its diverse role in several, very different diseases. ER-stress plays a role in such general processes like inflammation (reviewed by Yoshida 2007), e.g. airway epithelial cells synthesize a Leukocyte-adhesive hyaluronan matrix in response to ER-stress (Lauer *et al*. 2008). In myelodysplastic syndrome, down-regulation of Chop is caused by an aberrant methylation of its promoter (Wang YL *et al*. 2010; Lin *et al*. 2010). The UPR is also involved in cancer progression (reviewed by Healy *et al*. 2009). Indeed chemical induction of maladaptive ER-stress can potentially be a therapeutic option in anti-cancer therapies (Huang *et al*. 2011; Wang *et al*. 2011a). In transgenic mice, over-expression of XBP1(s) in B-cells and plasma cells enhances B-cell proliferative potential and activates known multiple myeloma-relevant pathways, leading to the development of multiple myeloma-like disease, mimicking many of the molecular, cellular, and clinical features of the human condition (Carrasco *et al*. 2007). Other examples for ER-Stress associated diseases are Joubert syndrome (Sayer *et al*. 2006b), rheumatic autoimmune disease ankylosing spondylitis (Lemin *et al*. 2007), inflammatory bowel disease (Kaser *et al*. 2008; Namba *et al*. 2009), artherosclerosis (Erbay *et al*. 2009; Hotamisligil 2010; Tabas 2009), non-alcoholic fatty liver disease (Puri *et al*. 2008), amyotrophic lateral sclerosis (Guégan, Przedborski 2003), Parkinson's disease or bipolar disorders.
As described above, maldaptive ER-stress was indicated to play a prominent role in IPF and represent a key trigger in the pathogenesis. The loss of cells by ER-Stress induced apoptosis is, with different levels of evidence and very diverse impact on the disease, linked to several other diseases. Importantly it can be caused by viral infection (reviewed by He 2006). It is involved in ß-cell death in Type I and Type II diabetes (Oyadomari *et al*. 2002; Oyadomari *et al*. 2001) (reviewed by Oslowski, Urano 2010; Araki *et al*. 2003; Eizirik *et al*. 2008; Lipson *et al*. 2006) but is not limited to the pancreas but probably also plays a role in Liver (reviewed by Kammoun *et al*. 2009), adipose tissue (reviewed by van der Kallen *et*

al. 2009) and kidney (Wu *et al*. 2010). However, in a mouse model of Type I diabetes CHOP knockout was not affecting development of diabetes (Satoh *et al*. 2011). Treatment of mice with LPS was inducing apoptotic ER-Stress in several cell types, including AECII and vascular epithelial cells, indicating a role for a maladaptive ER-stress in septic shock lungs (Endo *et al*. 2005). Maladaptive ER-stress can play a role in solid organ transplantation (reviewed by Pallet *et al*. 2010) for example it is involved in post-transplant injury in severely steatotic liver allografts (Anderson *et al*. 2011). Other examples for diseases associated with maladaptive ER-Stress are osteoarthritis (Takada *et al*. 2011) (reviewed by Kapoor, Sanyal 2009), Alzheimer's disease (Wiley *et al*. 2010; Wang *et al*. 2011b)(reviewed by Salminen *et al*. 2009), dilated cardiomyopathy (Hamada *et al*. 2004), hereditary Tyrosinemia Type I (Bergeron *et al*. 2006), proteinuric kidney disease (reviewed by Cybulsky 2010), ischemia (heart and neurons) (reviewed by Yoshida 2007) including chronic myocardial ischemia (Xin *et al*. 2011), alcohol induced liver disease and renal injury (reviewed by Yoshida 2007).

1.2.5 ER-stress in the alveolar epithelium

The alveolar epithelium consists of two different cell types, simply called type one (AECI) and type two (AECII). AECI cover most of the alveolar surface because they are widely stretched and therefore gauzy. Their main function is to cover the gas exchange area but they also play a role in water and ion transport (Dahlin *et al*. 2004; Dobbs *et al*. 1998; Johnson *et al*. 2002). The more abundant AECII have a rather conic shape. They do not only produce the surfactant (surface active agent) – film, which is required to reduce the alveolar surface tension and prevent the collapse of the alveoli, but also serve as the progenitor cells of the AECI (Brody, Williams 1992; Castranova *et al*. 1988). As secretory cells, especially the AECII normally have to handle a big ER-load.

1.3 Goals

ATF4, p50ATF6 and spliced XBP-1 are the three basic transcription factors of the UPR. In order to elucidate the downstream signalling pathways and cellular consequences of ER-stress, the first aim was to perform an over-expression of the above mentioned ER-stress signalling molecules ATF4, p50ATF6 and spliced XBP-1 in tumor derived MLE12 cells (generated in mice, harbouring the viral

oncogene simian virus 40 under transcriptional control of a promoter region from human SP-C). This approach included the co-transfection of two or three factors. Aim of i*n vitro* analysis was a broad general analysis of signalling pathways by transcriptome analysis (including the long and short-term effects of the ER-stress inducer TG), with verification of candidate genes via quantitative PCR (qPCR), miRNA-arrays and exon-arrays. The question in how far the over-expression of these factors can contribute to cell death (LDH-assay) via apoptotic signalling (induction of the pro-apoptotic mediator CHOP) in these cells had to be addressed. Knockdown of Atf4 and Atf6 via siRNA technology and investigation of its effects on Chop-induction represented another approach to address this question.

Furthermore, generation of transgenic mouse-lines for over-expression of the UPR-transcription factors and the pro-apoptotic mediator CHOP was a central goal. The over-expression in the transgenic mice had to be both inducible, to eliminate deleterious effects of ER-stress during lung development, and specific for the alveolar epithelium and, in there, the AECII-cells. This was gained by using a TET O7 system. The genes for ATF6 (NTD), ATF4 and spliced XBP-1 had to be cloned behind a TET O7-CMV promoter, which is inactive (operator-line). Oocyte injection and generation of founders was performed by EMBLEM [Transgenic animal facility of EMBL (Heidelberg)] (Monterotondo, Italy). This operator-line was crossed with a transactivator-line, which expresses the reverse tetracycline transactivator (rtTA) under control of the lung specific SP-C promoter. As a result, bi-transgenic mice were obtained, where the expression of the transgene is dependent on the application of Doxycycline (Dox), which binds to the rtTA and activates it. The breeding of the transgenic mice for experimental purposes, including the establishment of appropriate screening methods for transgene induction and homocygousity were technical aims of this work. A bidirectional vector system should be used to more easily monitor the transgene expression under *in vivo* conditions by in vivo imaging techniques. Dox induced mice should be investigated with regard to transgene expression and their potential downstream effects, especially eventually occurring fibrotic changes.

2. Materials

2.1 Chemicals

product	company
Acetic acid	Sigma Aldrich, Steinheim, Germany
Acrylamid / Bisacrylamid -solution (30 %, 37.5:1)	Carl Roth, Karlsruhe, Germany
Agarose	Carl Roth, Karlsruhe, Germany
Ampicillin (amp) Na-salt, molecular biology grade	Serva, Heidelberg, Germany
APS	Carl Roth, Karlsruhe, Germany
Ascorbic acid	Merck, Darmstadt, Germany
Brefeldin A	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Chloroform	Sigma-Aldrich, Steinheim, Germany
DMEM (Dulbecco's Modified Eagle Medium)	Invitrogen, Carlsbad, USA
DMEM F12	Invitrogen, Carlsbad, USA
Dharmafect 1	Thermo Fisher Scientific, Waltham, USA
dNTP (Deoxy ribonucleotide triphosphate) -mix	Thermo Fisher Scientific, Waltham, USA
MEM NEAA solution	Invitrogen, Carlsbad, USA
DMSO (Dimethyl-sulfoxide)	Merck, Darmstadt, Germany
EDTA (Ethylendiamin-tetra-acetic acid Ethylendinitrilo-N,N,N',N',-tetra-acetate)	Sigma-Aldrich, Steinheim, Germany
ß-estradiol	Sigma-Aldrich, Steinheim, Germany
Ethanol	Avantor, Deventer, Netherlands
Ethidiumbromide	Serva, Heidelberg, Germany
FCS (*fetal calf serum*)	PAA, Pasching, Austria
Glycerol	Sigma Aldrich, Steinheim, Germany
Glycine	Carl Roth, Karlsruhe, Germany
HCl	Merck, Darmstadt, Germany
H_2O_2 solution, 30 %	Carl Roth, Karlsruhe, Germany
Hydrocortison	Sigma-Aldrich, Steinheim, Germany

product	**company**
Isopropanol	Sigma-Aldrich, Steinheim, Germany
ITS-solution (100x)	PAN-Biotech, Aidenbach, Germany
Kanamycin (kan)	Carl Roth, Karlsruhe, Germany
KCl	Merck, Darmstadt, Germany
KH2PO4	Merck, Darmstadt, Germany
L-Glutamin solution (100x)	Invitrogen, Carlsbad, USA
Methanol	Sigma-Aldrich, Steinheim, Germany
NaCl	Carl Roth, Karlsruhe, Germany
Na2HPO4	Merck, Darmstadt, Germany
NaOH	Merck, Darmstadt, Germany
Optimem	Invitrogen, Carlsbad, USA
Sacharose	Carl Roth, Karlsruhe, Germany
2-mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
Sodium deoxy cholate	Fluka Chemie, Buchs, Switzerland
Sodium dodecyl sulphate	Merck, Darmstadt, Germany
Skimmed milk powder	Sigma-Aldrich, Steinheim, Germany
Tetramethylethylen-diamine N',N',N',N'-Tetra methyl diamine	Sigma-Aldrich, Steinheim, Germany
Thapsigargin	Invitrogen, Carlsbad, USA
TRIS (Tris(hydroxyl methyl)-amino methane)	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Bacto-Trypton	BD, Sparks, USA
Tween 20 (Polyoxyethylen(20) - sorbitanmonolaurate)	Sigma-Aldrich, Steinheim, Germany
Bacto Yeast extract	BD, Sparks, USA
Bacto Agar	BD, Sparks, USA
Aqua B.Braun	B.Braun, Melsungen, Germany
PBS (Phosphate-buffered Saline) (sterile)	PAA, Pasching, Austria
10x Trypsin / EDTA	Invitrogen, Carlsbad, USA
Developer	Agfa, Mortsel, Belgium
Fixer	Agfa, Mortsel, Belgium
Restore Western Blot Stripping Buffer	Thermo Fisher Scientific, Waltham, USA

S.O.C. Medium	Invitrogen, Carlsbad, USA

2.2 Solutions and Buffers

2.2.1 Solutions

10 x PBS	1.37 M NaCl 0.027 M KCl 0.081 M $Na_2HPO_4x2H_2O$ 0.018 M KH_2PO_4 pH 7.5 with NaOH
50 x TAE (TRIS / Acetic acid / EDTA buffer)	2 M TRIS 57.1 ml Acetic acid 10 % 0.5 M Na-EDTA, pH 8 water add 1 l
Water, dd-water (double distilled water)	deionised water
1 x Trypsin / EDTA	5 ml Stock solution + 45 ml PBS (sterile filtered)
10 x TBST	0.5 M NaCl 0.5 M TRIS pH 7.5 (HCl)
1 x TBST	10 x TBST diluted 1:10 with water

2.2.2 Loading buffers

5 x Loading buffer for Agarose gels	5 ml Glycerol 200 µl 50xTAE 400 µl Bromophenol blue solution (1 %)

	4.4 ml water	
4 x Loading buffer for Protein gels	TRIS / HCl (pH 6.8)	100 mM
	Glycerol	20 %
	SDS (sodium dodecyl-sulphate)	4 %
	Bromophenol blue	0.05 %
	for reducing gels:	
	2 -Mercaptoethanol	10 %

2.2.3 Running buffers

SDS-PAGE (Polyacrylamide Gel Electrophoresis) running buffer (2 l 10 x)	0.25 M TRIS 1.92 M Glycin 1 % SDS
Nucleic acid running buffer (1 x TAE)	1:50 dilution of 50 x TAE with water

2.2.4 SDS-PAGE-buffer

Stacking- gel buffer	0.625 M TRIS / HCl pH 6.8
Separating- gel buffer	1.125 M TRIS / HCl pH 8.8 30 % Sacharose

2.2.5 Western blot (WB)-buffer

Blocking buffer	5 % skimmed milk powder in 1 x TBST
2l Western transfer buffer	0.02 M TRIS 0.15 M Glycin 10 % methanol

2.2.6 Buffer for Protein extract

Lysis buffer	0.05 M TRIS 0.15 M NaCl 5 mM EDTA (Titriplex III) 1 % Triton X100 0.5 % Na-Deoxycholate (pH 7.4) 0.5 % 0.1 M PMSF solutions (freshly added)

2.2.7 Media

Mel188 medium	500 ml DMEM 50 ml FCS 5 ml NEAA (Sterile filtered)
MLE12 / Mle15 medium	500 ml DMEM F12 5 ml ITS solution 4 ml 1.25M HEPES (N-2-Hydroxy-ethylpiperazin-N-ethansulfonate) solution, pH 7.4 5 ml L-glutamin solution 10 ml FCS 500 µl 10 µM Hydrocortison solution 500 µl 10 µM ß-estradiol solution (Sterile filtered)
Starving medium	500 ml DMEM F12 5 ml ITS solution 4 ml 1.25M HEPES solution, pH 7.4 5 ml L-glutamin solution 0.5 ml FCS (Sterile filtered)
Transfection medium	50 % MLE12-Medium + 50 % Optimem

LB-medium (Luria Bertani-complete medium) / agar plates	5 g Bacto Tryptone 2.5 g Bacto Yeast Extract 5 g NaCl water add 500 ml 7.5 g Bacto Agar (for agar plates only) (autoclaved)
LB-amp-medium / agar plates	LB-medium / agar plates + 0.1 % amp (10 %) or kan (30 mg/ml) solution added at <50 °C

2.2.8 Enzyme buffer

product	**company**
NEB2	New England BioLabs, Ipswich, USA
NEB3	New England BioLabs, Ipswich, USA
NEB4	New England BioLabs, Ipswich, USA
Ligase buffer	New England BioLabs, Ipswich, USA Promega, Madison, USA

2.3 Length markers

product	**company**
page ruler (for Protein gels, pre stained)	Thermo Fisher Scientific, Waltham, USA
BenchMark (for Protein gels, pre stained)	Invitrogen, Carlsbad, USA
SmartLadder (for nucleic acid gels)	Eurogentec, Seraing, Belgium

2.4 Kits

product	**company**
DNeasy blood and tissue kit	QIAgen, Hilden, Germany
RNeasy plus mini kit	QIAgen, Hilden, Germany
QIAshredder	QIAgen, Hilden, Germany

product	company
ECL plus western blotting detection kit	GE healthcare, Buckinghamshire, UK
QIAquick Gel Extraction kit	QIAgen, Hilden, Germany
QIAquick PCR-purification kit	QIAgen, Hilden, Germany
BCA Protein Assay kit	Thermo Fisher Scientific, Waltham, USA
Cytotoxicity detection kit (LDH)	Roche, Basel, Switzerland
iQ SYBR Green Supermix	BioRad, Hercules, USA
HotStar Taq DNA Polymerase	QIAgen, Hilden, Germany
HotStar high fidelity DNA Polymerase	QIAgen, Hilden, Germany
Omniscript RT (reverse transcription) Kit	QIAgen, Hilden, Germany
Phire Hotstart Polymerase	Thermo Fisher Scientific, Waltham, USA
Phire Hotstart Polymerase II	Thermo Fisher Scientific, Waltham, USA

2.5 Proteins

2.5.1 Restriction endonucleases

product	company
*Bsr*BI	New England BioLabs, Ipswich, USA
*Aat*II	New England BioLabs, Ipswich, USA
*Drd*I	New England BioLabs, Ipswich, USA
*Ase*I	New England BioLabs, Ipswich, USA
*Nhe*I	New England BioLabs, Ipswich, USA
*Not*I	New England BioLabs, Ipswich, USA
*Xma*I	New England BioLabs, Ipswich, USA
*Sal*I	New England BioLabs, Ipswich, USA

2.5.2 Antibodies

target	dilution WB / IHC	conditions WB	antibody	company
Atf4	1:1000 / 1:500 and 1:1000	reducing	sc-200 Rabbit pAb	Santa cruz, Santa Cruz, USA / Aviva, San Diego, USA
Atf6	1:2000 / 1:200	reducing	ab 37149 Rabbit pAb	Abcam, Cambridge, UK
Xbp1	1:1000 / 1:50	reducing	sc-7160 Rabbit pAb	Santa cruz, Santa Cruz, USA
BIP	1:100000 / 1:750	reducing	368673 Rabbit pAb	Merck, Darmstadt, Germany (Calbiochem)
Cleaved Caspase3	1:500 / 1:50	reducing	2305 Rabbit pAb	Trevigen, Gaithersburg, USA
ß-actin	1:25000 / -	reducing	Ab8227 Rabbit pAb	Abcam, Cambridge, UK
Chop	1:200 / 1:50	non-reducing	sc-793 Rabbit pAb	Santa cruz, Santa Cruz, USA
Pro SP-C	- / 1:750	-	AB3786 Rabbit pAb	Merck, Darmstadt, Germany (Millipore)
Rabbit	1:1000 / -	secondary antibody	Swine anti rabbit, HRP (horseradish peroxidase)	Dako, Glostrup, Denmark

2.5.3 Other Proteins

product	company
Ribonuclease (RNase) -inhibitor	Roche, Basel, Switzerland
T4 DNA ligase	New England BioLabs, Ipswich, USA
T4 DNA ligase	Promega, Madison, USA
Bovine serum albumin (BSA) 10mg/ml solution	New England BioLabs, Ipswich, USA
Albumin Fraktion V (BSA)	Carl Roth, Karlsruhe, Germany

2.6 Primers

Primers were ordered at Metabion (Planegg-Martinsried, Germany). Unless mentioned otherwise, the primers were self designed with or without the aid of primer-blast software.

2.6.1 Amplification primers

name	sequence
ATF4-FOR-AMPL	CACGAGACGAAATCCAGCA
ATF4-REV-AMPL	TACAGCAAACACAGCAACAC
ATF6-FOR-AMPL	TCACCCATCCGAGTTGTGAG
2nd-ATF6-REV-AMPL	CATTATAAATGCTAATACTATCAT
CHOP-AMPL-FORW	GTGTTCCAGAAGGAAGTGCA
CHOP-AMPL-REV	CAAGCCGAGCCCTCTCCT

2.6.2 Cloning primers

name	sequence
ATF4-FORW-CLON-NheI-Koz	GATGCTAGCgccaccatgaccgagatgagcttc
ATF4-REV-CLON-NotI-pBI-L	GATGCGGCCGCctattacggaactctcttc
ATF4-REV-CLON-XmaI-pIRES2dsRed2	GATCCCGGGctattacggaactctcttctg
ATF6-FORW-CLON-NheI-Koz	GATGCTAGCgccaccatggagtcgccttttag
ATF6-REV-CLON-NotI-pBI-L	GATGCGGCCGCCTAacagacagctcttcgctttg

name	sequence
ATF6-REV-CLON-XmaI-pIRES2dsRed2	GATCCCGGGCTAacagacagctcttcgctttg g
Xbp1-FORW-CLON-NheI-Koz	GATGCTAGCgccaccatggtggtggtg
Xbp1-REV-CLON-NotI-pBI-L	GATGCGGCCGCttagacactaatcagctgg
Xbp1-REV-CLON-XmaI-pRES2dsRed2	GATCCCGGGttagacactaatcagctgggg
CHOP-FORW-CLON-NotI-Koz	GATGCGGCCGCgccaccatggcagctgag tccct
CHOP-REV-CLON-SalI-pBI-L	GATGTCGACtcatgcttggtgcaggct

Restriction site- appendix
Kozak- sequence
Translation start
Complementary part

2.6.3 Screening primers

name	sequence	source
Luc-RT-FORW	CCCAACACCGGCATAAAGAA	own design
Luc-RT-REV	TCGGTTGGCAGAAGCTATGA	own design
Luc-RT-PB-FORW	GTGTTCGTCTTCGTCCCAGT	own design
Luc-RT-PB-REV	ATGTAAACAATCCGGAAGCG	own design
SP-C rtTA –FORW	GACACATATAAGACCCTGGTCA	(Tichelaar *et al.* 2000)
SP-C rtTA –REV	AAAATCTTGCCAGCTTTCCCC	(Tichelaar *et al.* 2000)
pBI-L-Seq-FORW	GGTGGGAGGCCTATATAAGC	own design
pBI-L-Seq-REV	CAACACCCTGAAAACTTTGC	own design
pIRES2dsRed2-Seq-FORW	GCCCCATTGACGCAAATGG	own design
pIRES2dsRed2-Seq-REV	CCCTAGGAATGCTCGTCAAG	own design
HMBS-E4-FORW	CACCACGGGAGACAAGATTC	own design
HMBS-E4-REV	GGGCGTTTTCTAGCTCCTTG	own design

2.6.4 qPCR primers

name	target	sequence	source	PCR conditions
mß-AKTIN-FORW	murine Actb	CTACAGCTTCACCACCACAG	(Korfei *et al*. 2008)	standard
mß-AKTIN-REV	murine Actb	CTCGTTGCCAATAGTGATGAC	(Korfei *et al*. 2008)	standard
ß-MICRO-FORW	murine B2m	GCTATCCAGAAAACCCCTCAA	(Schmittgen, Zakrajsek 2000)	standard
ß-MICRO-REV	murine B2m	CATGTCTCGATCCCAGTAGACGGT	(Schmittgen, Zakrajsek 2000)	standard
HMBS-rt-Forw	murine Hmbs	CAAGAGTATTCGGGGAAACCTCAA	own design	standard
HMBS-rt-Forw	murine Hmbs	CAGGATCGTGCAACACACTCACTA	own design	standard
MU-Xbp1-ACO-Forw	murine Xbp1(s)	GCTTTTACGGGAGAAAACTC	(Acosta-Alvear *et al*. 2007)	standard / touchdown
MU-Xbp1-ACO-Rev	murine Xbp1(s)	GCCTGCACCTGCTGCG	(Acosta-Alvear *et al*. 2007)	standard / touchdown
ATF4-RT-Forw	murine Atf4	TTTGACAGCTAAAGTGAAGACTGA	own design	standard
ATF4-RT-Rev	murine Atf4	GCTTCTTCTGGCGGTACCTA	own design	standard
ATF6-RT-Forw	murine Atf6	AATGCCAGTGTCCCAGCAA	own design	standard
ATF6-RT-REV	murine Atf6	GCGCAGGCTGTATGCTGA	own design	standard
CHOP-FORW	murine Ddit3	CTAGCTTGGCTGACAGAGGA	own design	standard
CHOP-REV	murine Ddit3	CCTGGGCCATAGAACTCTGA	own design	standard
ATF3-FORW-RT-PB	murine Atf3	ACAACAGACCCCTGGAGATG	own design	standard

name	target	sequence	source	PCR conditions
ATF3-REV-RT-PB	murine Atf3	CCTTCAGCTCAG CATTCACA	own design	standard
VCP-FORW-RT-PB	murine Vcp	AGGTGCCACAAG TAACCTGG	own design	standard
VCP-REV-RT-PB	murine Vcp	GCCAGTAAGGTT TTCCCACA	own design	standard
EDEM-FORW-RT-PB	murine Edem1	TGGAATTTGGGA TTCTGAGC	own design	standard
EDEM-REV-RT-PB	murine Edem1	CATAGAAGGAAT CCAGCCCA	own design	standard
GRP94-FORW-RT-PB	murine Hsp90b1	CAGAGACTGTTG AGGAGCCC	own design	standard
GRP94-REV-RT-PB	murine Hsp90b1	CTTTGGATGGTC TCTGCCAT	own design	standard
PPIB-FORW-RT-PB	murine Ppib	TTTTGCTGCCCG GACCCTCC	own design	standard
PPIB-REV-RT-PB	murine Ppib	TCCTGTGCCATC TCCCCTGGT	own design	standard
BIP-FORW-RT-PB	murine Hspa5	TGGGAGGAGTC ATGACAAAA	own design	standard
BIP-REV-RT-PB	murine Hspa5	GGGGTCGTTCAC CTTCATAG	own design	standard
NFE2l2-FORW	murine Nfe2l2	GACTCGGTCCAG CGCAGTCG	own design	standard
NFE2l2-REV	murine Nfe2l2	TTCAGGCTCGGG GGCAGGTT	own design	standard
BAD-FORW	murine Bad	CCACCAACAGTC ATCATGGA	own design	standard
BAD- REV	murine Bad	AACTCATCGCTC ATCCTTCG	own design	standard
DNAJC10 – FORW	murine Dnajc10	AGCTGGGGTCTT GGATTTTT	own design	standard
DNAJC10 – REV	murine Dnajc10	CGGCTCTCACTT TTCCTTTG	own design	standard

name	target	sequence	source	PCR conditions
ATG12 -FORW	murine Atg12	AACAAAGAAATG GGCTGTGG	own design	standard
ATG12 – REV	murine Atg12	GAAGGGGCAAA GGACTGATT	own design	standard
CHKB –FORW	murine Chkb	TGACATCCAGGA AGGAAACA	own design	standard
CHKB – REV	murine Chkb	TCTAGTGGGGTA GTCCGTGG	own design	standard
Nf Y A –FORW	murine Nfya	AGTCAGTGGAG GCCAGCTTA	own design	standard
Nf Y A – REV	murine Nfya	CCAGGAGGCAC CAACTGTAT	own design	standard
hAKTIN-FORW	human Actb	ACCCTGAAGTAC CCCATCG	(Korfei *et al*. 2008)	standard
hAKTIN-REV	human Actb	CAGCCTGGATAG CAACGT	(Korfei *et al*. 2008)	standard
hPPIB-FORW	human Ppib	CCATCGTGTAAT CAAGGACTTC	(Korfei *et al*. 2008)	standard
hPPIB-REV	human Ppib	TAGCCAGGCTGT CTTGACTG	(Korfei *et al*. 2008)	standard
hEDEM-FORW	human Edem1	CCAAGACAGGG ATTCCATATCC	(Korfei *et al*. 2008)	standard
hEDEM-REV	human Edem1	TCACGACATTGC CTAGTAATCC	(Korfei *et al*. 2008)	standard
hCHOP-FORW	human Ddit3	AAAGATGAGCGG GTGGCAGCG	own design	64 °C
hCHOP-REV	human Ddit3	TCCCAGCTGACA GTGTCCGAAGA	own design	64 °C

2.7 Cells

product	company
Mle12	LGC standards, Wesel, Germany
Mle15	kindly provided by Tim Weaver, Cincinnati
Mel188	kindly provided by Tim Weaver, Cincinnati
One shot Top10 chemically competent cells [*E. coli (Escherichia coli)*]	Invitrogen, Carlsbad, USA

2.8 Plasmid vectors

name	source
pBI-L	Clontech, Saint-Germain-en-Laye, France
pIRES2dsRed2	Clontech, Saint-Germain-en-Laye, France
pEGFP-N1	Clontech, Saint-Germain-en-Laye, France
TETO/CMV/pUC19-Xbp1	Tim Weaver, Cincinnati
Atf4- pBI-L	own construct
Atf6- pBI-L	own construct
Xbp1- pBI-L	own construct
Chop- pBI-L	own construct
Atf4- pIRES2dsRed2	own construct
Atf6- pIRES2dsRed2	own construct
Xbp1- pIRES2dsRed2	own construct

2.9 siRNAs

All from Thermo Fisher Scientific, Waltham, USA

target	name	target sequence
Atf4	siGENOME siRNA D-042737-01	AGAUGAGCUUCCUGAACA
Atf4	siGENOME siRNA D-042737-04	AGCAUUCCUUUAGUUUAG
Atf6	siGENOME siRNA D-044894-03	AGUAUGGGUUCGGAUAU
Atf6	siGENOME siRNA D-044894-02	CAAUUGUAUUACCAGCAA
NT	siGENOME non-Targeting siRNA 1	

2.10 Machines and tools

product	company
Autoclave	HMC Europe, Engelsberg, Germany
Axiovert 25 (microscope)	Carl Zeiss Micro Imaging, Jena, Germany
Balances	
PB 801	Mettler Toledo, Greifensee, Switzerland
AB54	Mettler Toledo, Greifensee, Switzerland
Barnstaed Nanopure (deionized water supply)	Thermo Fisher Scientific, Waltham, USA
BioDoc II [Preparative UV (ultra violet) light -table]	Biometra, Göttingen, Germany
Canon Powershot A570 IS (CCD-camera)	Canon, Tokyo, Japan
Centrifuges	
Univapo 150H (vacuum centrifuge) and pump (vacuubrand RC6)	Montreal Biotech inc., Norwalk, USA Vacuubrand, Wertheim, Germany
Micro 200R (cooling centrifuge)	Hettich, Tuttlingen, Germany
Varifuge 3.0R (cooling centrifuge)	Heraeus, Hanau, Germany
Centrifuge 5426	Eppendorf, Hamburg, Germany
chambers for agarose-gel electrophoresis	Kreutz Labortechnik, Reiskirchen, Germany
Electric pipette holders	
Eppendorf Easypet	Eppendorf, Hamburg, Germany
Pipetus	Hirschmann Laborgeräte, Eberstadt, Germany
Gel Doc XR+ (Gel imager)	Biorad, Hercules, USA
Heating block VLM EC2	VLM, Bielefeld, Germany
Heraeus function line (CO_2- incubator)	Heraeus, Hanau, Germany HiClave HV50
HeraSafe (sterile bench)	Heraeus, Hanau, Germany
iCycler with MyiQ detection system (for qPCR)	Biorad, Hercules, USA
In vivo imaging system (IVIS),	

product	**company**
Night Owl II	Berthold Technologies, Bad Wildbad, Germany
Labogaz 206 (Bunsen burner)	Coleman, Wichita, USA
Micro pipettes	
Eppendorf reference (10 µl)	Eppendorf, Hamburg, Germany
Eppendorf reference (200 µl)	Eppendorf, Hamburg, Germany
Eppendorf reference (1000 µl)	Eppendorf, Hamburg, Germany
Microwave oven Severin 900	Severin, Sundern, Germany
Mini-Protean 3 Cell and Tetra Cell Electrophoresis chambers (for PAGE)	Biorad, Hercules, USA
MIRAX DESK (digital slide scanner)	Carl Zeiss Micro Imaging, Jena, Germany
MJ-Mini (PCR-cycler)	Biorad, Hercules, USA
Neubauer Haemocytometer	Optik Labor, Bad Homburg, Germany
Power supplies for Electrophoresis	
EV231	Consort, Turnhout, Belgium
E452	Consort, Turnhout, Belgium
P30	Biometra, Göttingen, Germany
PowerPac Basic	Biorad, Hercules, USA
Unidentified model	unknown
pH-meters	
pH330	WTW, Weilheim, Germany
HI221	Hanna instruments, Woonsocket, USA
pump (cell culture)	Ilmvac, Ilmenau, Germany
RH basic 2 (magnetic stirrer)	IKA-Werke, Staufen im Breisgau, Germany
Semi Dry Blot (Trans-Blot SD) blotting machine	Biorad, Hercules, USA
Severin folio (sealing machine)	Severin, Sundern, Germany
Shaker	
Vibrax-VXR with VX7	IKA-Werke, Staufen im Breisgau, Germany
Duomax 1030	Heidolph, Schwabach, Germany
Stuart mini gyro-rocker	Bibbi scientific, Stone, UK
Shaking incubators	
OV3	Biometra, Göttingen, Germany
unidentified model	Infors HT, Bottmingen, Switzerland

product	company
Spectrophotometers	
Uvikon 922	Biotek instruments, Winooski, USA
Nanodrop 2000C	Thermo Fisher Scientific, Waltham, USA
Spectrafluor Plus (ELISA-reader)	Tecan, Männedorf, Switzerland
Vortexer	
VWR VV3	VWR, Radnor, USA
Vibrax-VXR with VX1	IKA-Werke, Staufen im Breisgau, Germany
Precellys 24 (tissue homogenizer)	Bertin Technology, Montigny-le-Bretonneux, France

2.11 Software

name	source
EBI Pairwise Sequence Alignment	http://www.ebi.ac.uk/Tools/psa
EMBOSS Transeq	http://www.ebi.ac.uk/Tools/emboss/transeq/ index.html
Image lab (for Gel Doc)	Biorad, Hercules, USA
Iq5 Optical system software, version 2	Biorad, Hercules, USA
IVIS software (WinLight32)	Berthold Technologies, Bad Wildbad, Germany
Mirax viewer	Carl Zeiss Micro Imaging, Jena, Germany
NEB-cutter 2.0	http://tools.neb.com/NEBcutter2
Primer-Blast	http://www.ncbi.nlm.nih.gov/tools/primer-blast
Reverse-complement	http://www.bioinformatics.org/sms/rev_comp.html
Uniprot	http://www.uniprot.org

2.12 Consumables

product	company
Amersham Hybond-P [polyvinylidene fluoride (PVDF) –membrane]	GE healthcare, Buckinghamshire, UK
Amersham Hyperfilm ECL	GE healthcare, Buckinghamshire, UK
Aluminium foil	Carl Roth, Karlsruhe, Germany
Campingaz C206 (gas cartridges for Bunsen-burner)	Coleman, Wichita, USA
C-Chip (One-way Neubauer counter)	NanoEnTek, Seoul, Korea
Cell culture dishes (10cm, 6-well, 12-well)	Sarstedt, Nümbrecht, Germany Greiner Bio-One, Kremsmünster, Austria
Centrifugation tubes (15 and 50 ml)	Sarstedt, Nümbrecht, Germany BD, Sparks, USA
forceps, spattle, spoons	manufacturer unknown
Glassware	Carl Roth, Karlsruhe, Germany
one-way plastic pipettes (10ml and 25ml)	Sarstedt, Nümbrecht, Germany
one way-scalpel	Feather Safety Razor, Osaka, Japan
Pipette tips 10 µl, 100 µl, 1000 µl	Sarstedt, Nümbrecht, Germany
Pipette tips (filtered) 10 µl, 100 µl, 1000 µl	Nerbe Plus, Winsen / Luhe, Germany
microseal ´B´ Film (cover films for qPCR-plates)	Biorad, Hercules, USA
Microtest plates 96-well Flat Bottom (microtiter plates)	Sarstedt, Nümbrecht, Germany
Millipore Steritop Filter Units 33 and 45 mm (sterile filters)	Merck, Darmstadt, Germany
Nobaglove (Latex gloves)	NOBA, Wetter, Germany
Nunc Sealing tapes (cover films for microtiter plates)	Thermo Fisher Scientific, Waltham, USA
Parafilm (M)	Pechiney Plastic Packing, Menasha, USA
Pasteur pipettes	VWR, Radnor, USA
PCR-reaction tubes	Sarstedt, Nümbrecht, Germany
Peha Soft (Nitrile gloves)	Hartmann, Heidenheim, Germany

product	**company**
Petri dishes	Carl Roth, Karlsruhe, Germany
qPCR-plates	
AB-1400/w	Thermo Fisher Scientific, Waltham, USA
iCycler iQ PCR-Plates	Biorad, Hercules, USA
Reaction tubes (1.5 and 2 ml)	Sarstedt, Nümbrecht, Germany Eppendorf, Hamburg, Germany
Rotilabo syringe filters	Carl Roth, Karlsruhe, Germany
Syringe, 20 ml	B.Braun, Melsungen, Germany
weighing paper	Machery-Nagel, Düren, Germany
Whatman 3 mm filter paper (blotting paper)	GE healthcare, Buckinghamshire, UK

2.13 Mouse lines

name	**function and features**	**source / reference**
FVB/N	WT (wild type)-inbred line	EMBLEM and Charles River Laboratories (CRL) (Kisslegg, Germany)
SP-C-rtTA	Transactivator line for production of rtTA in AECII, FVB/N background	(Tichelaar *et al*. 2000)
Atf4-pBI-L	Line for conditional over-expression of Atf4, FVB/N background	own line
Atf6-pBI-L	Line for conditional over-expression of Atf6, FVB/N background	own line
Xbp1-pBI-L	Line for conditional over-expression of Xbp1(s), FVB/N background	own line

3. METHODS

3.1 General methods

3.1.1 Working with RNA

RNA can easily be destroyed by ubiquitous RNases. All works with RNA were therefore performed with gloves and RNase-free pipette tips. RNA solutions were produced and diluted with RNase free water (QIAgen, either from the RNeasy + Kit or the Reverse transcription Kit). To further reduce the activity of RNases, works with RNA were generally performed on ice, unless this possibility was excluded by experimental necessities. RNAs were either stored at -20 or -80 °C, if long term storage was necessary.

3.1.2 Centrifugation

Centrifugations in volumes $\leq$ 2 ml were performed in an Eppendorf (Centrifuge 5426) table top centrifuge or a Hettich Micro 200R cooling centrifuge. For bigger volumes a Heraeus Varifuge 3.0R cooling centrifuge was used. Vacuum centrifugation was performed in a Montreal Biotech Univapo 150H centrifuge with an attached Vacuubrand RC6 vacuum pump. The same centrifuge was also utilized to spin down reaction mixes in qPCR plates.

3.1.3 Clonal amplification, preparation and storage of plasmid DNA

For clonal amplification of plasmids, chemo-competent *E. coli* cells (Top10, invitrogen) were transformed with 0.5 µl of the concentrated plasmid solution or 5 µl of a ligation mix following the enclosed protocol. 50 µl of the bacterial culture were directly spread on an LB-agarose plate with 0.1 % amp (10 %) or kan (30 mg/ml) with the help of a Drigalski-spatula (self made out of a glass pole). If higher bacterial concentrations were required, the remaining bacteria were centrifuged down (1000g, 5 min), resuspended in 50 µl LB-medium and then spread on a second LB-agarose plate. The plates were pre-dried for 1 h and then incubated bottom-up at 37 °C in a Biometra OV3 or Infors HT (unidentified model) incubator over night (ON). Clones picked from these plates were incubated in small volumes of LB-medium with the appropriate antibiotic [96well plate, 37 °C,

220 rpm (revolutions per minute), 5 h]. If required, 5 µl of this pre-culture were taken out for Polymerase Chain Reaction *(*PCR)-screening.
50-200 µl *E. coli* from this pre-culture or alternately directly from existing cryo stocks containing the plasmid of interest, were transferred into 200 ml LB-medium with amp or kan (depending on the resistance cassette of the plasmid, concentrations as described in the material chapter) in a 500 ml Erlenmeyer tube. The culture was then incubated in an Infors HT (unidentified model) rotation shaker at 37 °C and 220 rpm ON. If required, 800 µl of this bacterial solution were mixed with 200 µl glycerol (sterile) and frozen at -80 °C as a new bacterial stock. The remaining culture was transferred into 50 ml Falcon (centrifugation tube) and spinned down 30 min at 4000 rcf (relative centrifugal force). The plasmid was then isolated with a QIAgen Endofree plasmid maxiprep-Kit following to the manufacturers instructions except for the precipitation of the plasmid, which was done by centrifugation for 1 h at 4000 rcf. The plasmid-pellet was resuspended in 100 µl dd-water and stored at -26 °C in non-pyrogenic 1.5 ml tubes. Plasmid DNA was kept sterile by working next to a Bunsen-burner or in a sterile bench.

3.1.4 Culturing, melting and freezing of different cell lines

MLE12, MLE15 and MEL188 (Human SK MEL-188 Melanoma Cells) cells were kept in 10 cm cell culture dishes with 10 ml MLE12-Medium (for MLE12 and MLE15) or MEL188-Medium. The cells were cultured in an incubator at 37 °C with 5 % CO_2. For maintaining the culture, the cells were split 1:10 (MLE12 and MEL188) or 1:5 (MLE15) on Mondays and Fridays. For splitting, the medium was sucked away with a sterile Pasteur pipette attached to a vacuum pump. Then the cells were washed with 10 ml PBS and harvested by incubating for 1-5 min in the 37 °C-Incubator with 3 ml Trypsin / EDTA followed by knocking the plates to a solid surface until the cells were de-attached. Then 7 ml culture medium was added and they were transferred to a 50 ml Falcon. The cells were spinned down (5 min; 1000 rcf). The medium with the Trypsin was sucked of as described above and the cells were resuspended in 10 ml fresh culture medium. The required volume (e.g. 1 ml for splitting 1:10) was transferred to a fresh plate.
MLE12-cells were used no longer than passage fifteen (after purchasing), MLE15 and MEL188 cells no longer than passage 30.
When cell-cryostocks from liquid nitrogen had to be thawed, the cells were pre-warmed in the hand and transferred completely into culture medium as soon

as possible. After 1-2 days the culture medium was changed, and then the cells were split normally when they reached full confluence.
When cryo-conservation of MLE12 or MLE15 cells was required, cells were harvested in the described way. The pellet was overlaid with 500 µl Medium 1 (culture medium and 0.2 M HEPES-Buffer; 9:1) and incubated on ice for 5 min. Then the cells were resuspended and transferred into a cryo vial with a pipette. 500 µl Medium 2 (Medium 1 and DMSO; 4:1) was added. The cells were incubated 5 min on ice, then packed into styropor and incubated 2-3 h at -26 °C and ON at -80 °C. Then the vials were unpacked and transferred into liquid nitrogen for long term storage. Harvested MEL188 were resuspended in a special freezing medium (Culture medium, FCS, DMSO; 6:3:1) and then frozen as described for the other cells.
All works with open cells were performed under sterile conditions in a cell culture bench.

3.1.5 Counting cells in broncho-alveolar lavage (BAL)

To count cells in murine BAL, 10 µl of cells were transferred into a disposable haemocytometer (c-chip) or a Neubauer-haemocytometer then the cells in fife large squares were counted under a microscope. The average number of cells per square was multiplied by 10000 resulting in cells / ml.

3.1.6 Preparation of enriched mRNA

For mRNA-microarrays and qPCR, enriched mRNA was prepared using the QIAgen RNeasy Plus mini-kit according to the standard procedure in the applications manual. Cell culture cells were harvested with 600 µl buffer RLT+ and homogenized with a QIAshredder. The elution of the mRNA was done with RNase free water (30-50 µl, as appropriate). RNA prepared this way was depleted from DNA (by cDNA-eleminator columns) and enriched for bigger RNAs because small RNAs do not bind strongly to the columns under these buffer conditions.

3.1.7 Reverse transcription (RT) of mRNAs

For the preparation of cDNA, equal amounts of enriched RNA (typically 2 µg) per sample were subjected to reverse transcription with the Omniscript RT kit

(QIAgen) with oligo-dT-primers. 6µl of the following master mix was added to the appropriate amount of RNA:

Master mix:	
10 x Buffer	2 µl
dNTPs	2 µl
Oligo-dT primers	0.5 µl
RNase Inhibitor	0.5 µl
Reverse transcriptase	1 µl

Before adding RNA and master mix, RNase-free H_2O was subjoined to achieve a total reaction volume of 20 µl for each sample. The samples were incubated 10 min at RT (room temperature) for primer annealing and then 65 min in a thermocycler at 37 °C for cDNA synthesis. For convenience, the concentration of the RNA-template was used as the concentration of the product cDNA in all subsequent methods. However, the true cDNA content of the sample could be lower because the template is not pure mRNA and the efficiency of the RT is never 100 %.

3.1.8 Preparation of protein-extracts

To produce raw protein extract from cells an appropriate amount of lysis-buffer (e.g. 100 µl for a well 6-well) was added to the adherent cells, which were washed with PBS in advance. After that, the plate was shock-frozen in liquid nitrogen and melted at RT three times and then transferred into a centrifuge tube. Alternatively cells were scratched of the plate after addition of the lysis buffer, resuspended and transferred into a centrifuge tube, which was then treated with liquid nitrogen accordingly. Protein raw-extracts from murine lung tissue were either produced by grinding in liquid nitrogen with mortar and pestle or by using a Bertin Technology Precellys 24 tissue homogenizer.
When using mortar and pestle, lysis buffer (e.g. 1 ml) was added to the fine, frozen lung powder. The mixture was transferred into 1.5 ml tubes after melting and incubated on ice for additional 30 min.
When using the homogenizer, a frozen lung piece was put into a homogenization tube with ceramic beads and lysis buffer and then homogenized two times at 5000 rpm for 30 sec. To get clear extracts, the raw extracts were centrifuged at 20000 rcf for 10 min at 4 °C. The supernatants were stored at -26 °C or -80 °C.

3.1.9 Preparation of total RNA (for miRNA)

For miRNA arrays total RNA including the small RNAs is required. Total RNA was prepared with the QIAgen RNeasy Plus mini-kit according to appendix D of the applications manual. The different use of buffers (with more Ethanol) allows the binding and purification all RNAs, including the miRNA.

3.1.10 Estimation of protein concentrations (BCA)

Protein concentrations were estimated by BCA-assay. The BCA-assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and a subsequent reaction of bicinchoninic acid (BCA) with the reduced (cuprous) cation to a purple-coloured product which can be assessed by absorbance at 562 nm (Smith *et al*. 1985). The assay was performed according to the instructions manual of the kit. Proteins were diluted 1:5-1:20 in lysis-buffer as appropriate. 1500, 1000, 750, 500, 250, 125, 62.5, 31.25, 15.625 and 7.813 µg/ml BSA solutions (in lysis-buffer) served as reference standard. After incubating the protein solutions for 30 min at 37 °C with the assay reagent, the protein concentrations were estimated in a Tecan SpectrafluorPlus microtiter plate reader at 560 nm. The results were multiplied with the appropriate dilution factor.

3.1.11 Measuring nucleic acid concentrations

Nucleic acid concentrations were either measured in a Biotek instruments Uvikon 922 cuvette-spectrophotometer or in a Thermo Fisher Scientific Nanodrop 2000C spectrophotometer. When the cuvette-spectrophotometer was used, the nucleic acids had to be pre diluted 1:50 to 1:100 and put into a quartz-cuvette. The nucleic acid concentration was calculated from the optical density (OD) at 260 nm:

DNA: 1 OD = 50 µg/ml
RNA: 1 OD = 40 µg/ml

The estimation in Nanodrop follows the same principles. However, measurement and calculations are done automatically by the machine. The Nanodrop also allows direct measurement out of 2 µl (or less) of a sample without any dilution (concentration range 2 ng/µl to 15 µg/µl), as it adjusts the thickness of the sample layer for the measurement.

3.1.12 Precipitation and washing of DNA

DNA was precipitated by addition of 10 % sodium acetate solution (3 M, pH 5) and three volumes of 100 % ethanol followed by 2 h incubation at -26 °C. Then the DNA was pelleted by centrifugation (20000 rcf, 4 °C, 20 min). The liquid was removed with a pipette and the pellet was washed by addition of 500 µl 70 % Ethanol and subsequent centrifugation (20000 rcf, 4 °C, 5 min). After removing the ethanol, the DNA pellet was dried (open tube, RT, 3-15 min).

3.1.13 Preparation of genomic DNA

Genomic DNA from mouse tail clips (provided by CRL) was generally produced with the QIAgen DNeasy Blood and Tissue kit following the instructions of the manual. Genomic DNA from founder mice was provided as a pellet in 70 % ethanol (by EMBLEM) and spinned down, dried and resuspended in 500-1000 µl water before further use.

3.1.14 Poly Acrylamid Gel Electrophoresis (SDS-PAGE)

4 x Sample buffer (SB) was added to the protein extracts as required to achieve 1 x SB. Generally equal protein amounts of 20-50 µg were separated in SDS-PAGE. Depending on the target, they were either run reducing (with 10 % ß-mercaptoethanol in the sample buffer) or non-reducing. Prior to loading, the samples were denatured 10 min in boiling water and then chilled on ice. The samples were spinned down in a centrifuge and then loaded on self made SDS -polyacrylamide gels with a 9-15 % separating gel and a 4 % stacking gel. The proteins were separated at 15 mA (per gel).

3.1.15 Western blots and immunostaining of immobilized proteins

The gels from SDS-PAGE were used for WB analysis. The PVDF-membrane and blot-papers were cut to gel size. The membrane was activated in 100 % methanol for 2 min and then pre-incubated in transfer-buffer. After electrophoresis the gels were also pre-incubated in transfer-buffer. Then the blotting machine (Biorad) was set up (from bottom to top: three layers of blotting paper, membrane, gel, three layers of blotting paper) and the transfer was performed at 25 V for 90 min.

The membrane was then incubated in blocking-buffer for 1 h (on a shaker). Then the membrane was placed in a sealed plastic bag with about 12 ml antibody-solution per gel and incubated ON at 4 °C on a shaker. The membrane was washed in a plastic dish with 1 x TBST (4 x 15 min) and incubated with the appropriate peroxidase coupled secondary antibody (1:2000, RT, 1-2 h, shaking). The membrane was then washed six times with TBST as described above and placed in a cut-open plastic disposal bag for enhanced chemo-luminescence (ECL)-detection. In the darkroom 3 ml of freshly detection reagent (GE-Healthcare, ECL western blot detection system) were applied to the membrane, followed by 5 min incubation. The membrane was briefly dried between two paper towels and then placed in a cut-open disposal bag in a film cassette. Films were put on (and developed as described below) for different exposure times depending on the required signal intensity.

3.1.16 Developing films

The films were put on the blots in the dark. After variable times of exposure, they were transferred into developing solution (1 min), washed in water and transferred into the fixing solution for some minutes. The film was then washed in water again and dried with a hot air blower.

3.1.17 Immunohistochemistry (IHC)

IHC is based on the localization of antigens in tissue sections by labelled antibodies. These primary antibodies are then detected by enzyme-labelled secondary antibodies and visualized by the production of a coloured substrate by these enzymes.
For the purposes of this thesis 3 µm paraffin embedded serial sections of paraformaldehyde fixed mouse lungs were stained (either by me or under my supervision by Dirk Lohfink). Generally every second section was stained for pro-SP-C. Sections were deparaffinised at 60 °C for 1 h and then in xylol for 10 min. They were then rehydrated in alcohol baths with declining concentrations (99.6 %, 96 %, 80 %, 70 % and 50 %, each for 3 min) and kept in distilled water until further use.
Antigen retrieval was performed by denaturation in citrate buffer in a microwave oven (three times 5 min boiling at 600 W, interrupted by cooling down at RT for 25 min and refilling with aqua dest up to the original level).

The object carriers were then washed with 1 x PBS (3 x 2 min with shaking in staining bath), and quickly dried with paper towels without touching the slide.
The ZytoChem AP fast red kit or HRP- DAP kit were used for further steps. The sections were placed in a humid chamber and blocked for ten minutes with two drops of blocking solution, followed by washing as described above. Primary antibodies (purchased separately) were prepared in 3 % BSA solution in dilutions listed with the antibodies. The slides were covered with equal amounts (typically 100 µl) of this antibody solution and incubated for variable times (typically 1.5 h at RT). A negative control (without primary antibody) was included.
After another washing step, two drops of the biotinylated secondary broad-spectrum-antibody solution were put on each slide. After 15 min of incubation at RT and another washing step, two drops of Streptavidin-alkaline phosphatase solution (or HRP-solution for the DAP-kit) were added followed by another 15 min incubation and washing.
One pill of Fast Red (or two drops of DAP-solution for the DAP-kit) was freshly dissolved in a bottle of naphtol (provided) and two drops were put on each slide. The slides were incubated up to 20 min depending on the stained protein and the desired degree of staining, which was controlled under the microscope. The slides were washed in distilled water. Finally the slides were stained with haemalaun for 30 sec and washed under running tap-water. Sections were then mounted with glycerol mounting medium and cover slips and allowed to dry ON.

3.1.18 Microscopy

Stained sections were usually analysed by scanning with Mirax scanner (Carl Zeiss). The Mirax viewer software was used for making and analyzing snapshots. Alternately a Carl Zeiss Axiovert 25 microscope with light and fluorescence optic , was used to view slides as well as cell culture cells and an attached Canon Powershot A570 IS digital camera was utilized to produce images.

3.1.19 Agarose gel-electrophoresis

The DNA samples were mixed with loading buffer (final concentration 1 x) and loaded onto a self made one or 2 % agarose gel with 1 µg/ml ethidium bromide (except for ethidium bromide free gel extraction).
The separation was performed at 60-100 V until the desired distance of separation was reached. Due to their negative charge nucleic acids migrate towards the

anode. The migration distance of DNA in a gel depends mainly on its size but can vary for circular, especially super coiled DNA. The size of amplified DNA was determined by a DNA molecular weight standard (SmartLadder, Eurogentech). The agarose gel was visualized and photographed under UV light in a Biorad Gel Doc XR+ gel imager.
The agarose gel-electrophoresis was used to estimate DNA concentrations by comparing it with the marker, verifying product sizes from qPCR, identifying the products of PCR reactions and for gel purification of DNA-products with the QIAquick Gel Extraction Kit (according to the manual).

3.1.20 Polymerase chain reaction (PCR)

PCRs were performed for different purposes, firstly for the PCR-screening of the transgenic animals and of plasmid-vectors for the presence of transgene and transactivator, secondly as a preparation for the cloning of transgenes into different plasmid vectors. Depending on the purpose, different Polymerase-systems were used. For PCR-screenings either the QIAgen hot start polymerase kit or the Thermo Phire -polymerase system was used.

Reaction mix for QIAgen hot start polymerase:

reagent	**µl for 25 µl reaction**
10 x buffer	2.5
dNTP-Mix	0.5
Forward primer 10 pmol/µl	2.5
Reverse primer 10 pmol/µl	2.5
HotStar DNA Polymerase	0.25
DNA	1
Water	15.75

Reaction mix for Phire polymerase:

reagent	**µl for 10 µl reaction**
5 x Buffer	2 µl
Forward primer 10 pmol/µl	0.5 µl
Reverse primer 10 pmol/µl	0.5 µl
dNTPs	0.2 µl
Phire Polymerase	0.2 µl
Water	5.6 µl
Template	1 µl

PCR amplifications for cloning were performed with QIAgen HotStar HiFidelity PCR-Kit.

Reaction mix for QIAgen HotStar HiFidelity polymerase:

reagent	**µl for 50 µl reaction**
5 x Puffer (incl. dNTPs)	10
Forward primer 100 pmol/µl	0.5
Reverse primer 100 pmol/µl	0.5
HotStar HiFidelity DNA Polymerase	1
Template	(100 ng)
water	ad 50 µl

The PCR conditions were depending on the Polymerase System and on the target.

Conditions for PCR with luciferase primers and QIAgen polymerase:

hot start	1 x	20 min	95 °C
denaturing	30 cycles	30 sec	94 °C
annealing		30 sec	61 °C
elongation		60 sec	72 °C
final Elongation	1 x	10 min	72 °C

Primers: Luc-RT-FORW / Luc-RT-REV

Conditions for PCR with pBI-L insert-primers / dsRed-insert primers and QIAgen polymerase:

hot start	1 x	20 min	95 °C
denaturing	30 cycles	60 sec	94 °C
Annealing		60 sec	53 °C / 55 °C
Elongation		60 sec	72 °C
final Elongation	1 x	10 min	72 °C

Primers: pBI-L-Seq-FORW / pBI-L-Seq-REV; pIRES2dsRed2-Seq-FORW / pIRES2dsRed2-Seq-REV

Conditions for PCR with luciferase and SP-C-rtTA primers and Phire polymerase:

hot start	1 x	30 sec	98 °C
denaturing	30 cycles	5 sec	98 °C
annealing		5 sec	62 °C
elongation		20 sec	72 °C
final Elongation	1 x	1 min	72 °C

Primers: Luc-RT-FORW / Luc-RT-REV; SP-C rtTA –FORW / SP-C rtTA –REV

Basic conditions for PCR with QIAgen HotStar HiFidelity polymerase:

hot start	1 x	5 min	95 °C
denaturing	45 cycles	15 sec	94 °C
annealing		60 sec	53 °C
elongation		65 sec	72 °C
final Elongation	1 x	10 min	72 °C

These conditions were further optimized for the individual cloning-reactions as follows:
Pre-amplification of the Atf4-insert: elongation: 80 sec; annealing 51 °C
Pre-amplification of the Atf6-insert: elongation: 80 sec; annealing 48 °C
Xbp1-PCR with cloning Primers: annealing 55 °C

Primers: ATF4-FOR-AMPL / ATF4-REV-AMPL; ATF6-FOR-AMPL / 2nd-ATF6-REV-AMPL; CHOP-AMPL-FORW / CHOP-AMPL-REV; ATF4-FORW-CLON-NheI-Koz / ATF4-REV-CLON-NotI-pBI-L; ATF4-FORW-CLON-NheI-Koz / ATF4-REV-CLON-XmaI-pIRES2dsRed2; ATF6-FORW-CLON-NheI-Koz /ATF6-REV-CLON-NotI-pBI-L; ATF6-FORW-CLON-NheI-Koz / ATF6-REV-CLON-XmaI-pIRES2dsRed2; Xbp1-FORW-CLON-NheI-Koz / Xbp1-REV-CLON-NotI-pBI-L; Xbp1-FORW-CLON-NheI-Koz / Xbp1-REV-CLON-XmaI-pRES2dsRed2; CHOP-FORW-CLON-NotI-Koz; CHOP-REV-CLON-SalI-pBI-L

3.1.21 Quantitative PCR (qPCR) analysis

Quantitative PCR (qPCR) was performed for relative quantification of mRNA and genomic DNA (screening for homozygousity) levels. For each reaction the following mix was prepared on 96-well PCR-plates:

reagent	**µl for 20 µl reaction**
BIORAD Sybergreen Mix	10
Forward primer 10 pmol/µl	0.5
Reverse primer 10 pmol/µl	0.5
Water	8
Template (10 ng/µl)	Je 1 µl (10 ng)

The reaction mix was prepared as a master mix without the template. Per well, 19 µl of the mix were put on the PCR plate, then 1 µl of the respective DNA-template (10 ng/µl) were mixed in with a pipette. The plates were sealed with a Biorad microseal ´B´ film and centrifuged to remove air bubbles and concentrate the sample at the bottom of the plate. Then the plate was placed in a Biorad iCycler with MyiQ detection system and incubated according to the following protocol:

	1 x	3 min	95 °C
denaturing	40 cycles	15 sec	95 °C
annealing		30 sec	60 °C detection
	1 x	10 sec	melting curve analysis

For some targets, the conditions had to be adjusted. Individualized annealing temperatures are listed with the primers. The Xbp1(s) reaction sometimes produced multiple products. In those cases twenty touchdown-cycles were performed before the detection phase, where the annealing temperature was reduced in each step by 0.5 °C starting with 70 °C.

Quality control:
Results from qPCR were only accepted when they matched several quality standards. Firstly, for each individual cDNA sample of a –RT control had to be

negative. For that purpose 10 ng of the template RNA was taken as template for the same qPCR-reaction as the cDNA (either with the primers for a reference gene or for the transfected transgene). Samples were accepted when the cycle of threshold (CT) of those samples was at least three below the CT of its cDNA. Secondly, equal amplification efficiencies of the different samples from one reaction had to be guarantied. This was estimated by observation of the amplification curves in the region of the threshold. Only curves which appeared to be parallel were accepted. Thirdly the reference genes had to be equal. Samples in which a reference gene was three CT-values away from the mean were not accepted. Fourthly, the first derivation of the melting curve was observed. Reactions with more then one peak (representing multiple products or primer dimmers) were not accepted.
For some reactions additional quality controls were performed in advance: The first was the separation of the product on an agarose gel to check the product identity (by size). The second was the comparison of the CT of a reaction with 10 and 20 ng template to verify that a quantitative analysis with the used concentrations was possible.

Data analysis:
The primary results of a qPCR are the CT-values (the cycle-number in which the light signal reaches a certain threshold value which lies in the early exponential phase of the reaction). By subtraction of the CT-values from those of reference genes, smaller differences in template quality and concentration can be corrected. All qPCRs of murine cDNA were normalized against the mean CT of ß-actin, Hmbs and ß-microglobulin. GDNA qPCRs were normalized against Hmbs (Hmbs E4-primer) and human cDNAs were normalized with ß-Actin. When appropriate the delta CT (dCT) -values of a given treatment were further normalized against the untreated or control treatment (by subtraction of the control dCT from the target gene dCT), leading to the delta-delta CT-value (ddCT). Delta CT values only have a meaning when compared to one or more references, whereas ddCT values directly represent an up- or down-regulation.

3.1.22 Sterile work

Many methods had to be performed under sterile conditions therefore the following sterile techniques were performed for the indicated purposes.

technique	application
Autoclaving	Sterilization of LB-medium (before addition of Antibiotics) Bacterial and cell culture waste disposal sterilization of containers, pipette tips, etc. Sterilization of glycerol
Sterile filtration	Sterilization of cell culture media and reagents with Syringe-filters for volumes <100 ml and bottle top filter and vacuum pump for larger volumes
Disinfection with 70 % ethanol	general decontamination of working surfaces also preparation for sterile work; disinfection of pipettes
Work next to a running Bunsen-burner	Work with bacteria (except harvesting) and fresh bacterial culture media
Flaming with Bunsen Burner	local sterilization of glassware when working with bacteria

3.2 Cloning

This chapter serves as an overview over the method. Details are described in the chapter PCR and primer sequences are documented in the material part (2.6.2).

3.2.1 Overview and amplification of the inserts

The following constructs were cloned:

insert	reference	plasmid vector
Atf4	Uniprot Q06507 (sequence according to the indicated Q→K sequence conflict	pBI-L
Atf6 N-terminal domain	Uniprot Q8BZ84 nucleotides 1-1098 +tgt	pBI-L
Xbp1(spliced)	Uniprot O35426, isoform 2	pBI-L
Ddit3 (Chop)	Uniprot P35639	pBI-L
Atf4	Uniprot Q06507 (sequence according to the indicated Q→K sequence conflict	pIRES2dsRed2
Atf6 N-terminal domain	Uniprot Q8BZ84 nucleotides 1-1098 +tgt	pIRES2dsRed2
Xbp1(spliced)	Uniprot O35426, isoform 2	pIRES2dsRed2

The basis material for Atf4, Atf6 and Chop-constructs was murine cDNA. Template for spliced Xbp1 was the plasmid TETO/CMV/pUC19-Xbp1 (obtained from Prof. T. Weaver, Cincinnati). For Atf4, Atf6 and Chop a pre amplification of a larger sequence, containing the open reading frame was performed and then used as a template for the actual cloning PCR to enhance amplification efficiency and specificity of the product. The cloning primers (for all the constructs) were designed with the following features:
Forward primer composition:
- Six unspecific base pairs - sequence for the cleavage of a restriction endonuclease which is also present in the multiple cloning site of the plasmid but not in the insert – Kozak sequence – start codon and additional bases complementary to the insert sequence -
Reverse primer composition:
- Additional bases complementary to the downstream region of the insert sequence ending with the Stop codon - sequence for the cleavage of a restriction endonuclease which is also present in the multiple cloning site (downstream of the restriction site of the forward-primer) of the plasmid but not in the insert six unspecific bases -

3.2.2 Digestion of inserts and vectors

To enable forced orientation cloning, the inserts and the respective vectors were cut with the same two restriction endonucleases, leading to homologous single stranded ends of the vector and the insert (for that purpose only restriction sites for such endonucleases which produce “sticky” ends were chosen). To enable an optimal ligation in the next step the restriction cuts had to be as complete as possible. At the same time unspecific digestion had to be kept at a minimum. The two digestions were performed independently. After the first digestion the DNA was purified with the QIAgen PCR-purification kit (the matrix of the contained spin columns does not bind small fragments and the cut of ends of the primer sequences are therefore removed). The second digestion was purified with gel extraction (QIAgen gel extraction Kit). This allowed removing the larger cut out fragments from the MCS of the plasmid as well as it enabled the purification of the inserts from most unspecific amplification products.

3.2.3 Ligation of inserts and vectors

The linearized vectors and purified inserts were ligated in molar ratios of roughly 1:3. Two different ligase systems (depending on availability in the laboratory) were used for this step. The constructs containing Atf4, Atf6 and Xbp1 were ligated with NEB T4 DNA ligase, the Chop-construct with Promega ligase.

Ligation with NEB-ligase:

digested insert	**linearized plasmid**	**ligase**	**ligase buffer**	**water**	**reaction**
100 ng	25 ng	1 µl	2 µl	ad 20 µl	16 °C, ON

Ligation with Promega-ligase:

digested insert	**linearized plasmid**	**ligase**	**ligase buffer**	**water**	**reaction**
100 ng	25 ng	0.5 µl	1 µl	ad 10 µl	16 °C, ON

3.2.4 From ligation to vector

The ligated plasmids were then cloned into *E. coli*. The procedures of this step are described in the chapter “Clonal amplification, preparation and storage of plasmid DNA”. As indicated there, 5 µl of small bacterial cultures derived from the picked clones were used for a PCR screening. The method is described in the chapter “Polymerase chain reaction (PCR)” as screening for the insert of pIRES2dsRed2 of pBI-L respectively. However, for this special case, the template was 5 µl of a bacterial culture instead of 1 µl DNA-solution and the volume of the water in the reaction was adjusted accordingly. When big amounts of plasmids from maxipreps were available, an aliquot was send to GATC-Biotech for sequencing. The primers used for the PCR-screening of the insert were also used for sequencing in forward and reverse direction. The sequences were then compared to the expected insert sequence as obtained from a databank with the aid of different bioinformatics’ tools, available in the www (EBI Pairwise Sequence Alignment, EMBOSS Transeq, Uniprot, Reverse-complement).

procedure	name of the tool
Reverse complementation of the sequence obtained with the reverse primers	Reverse complement
Comparison of sequences	EBI Pairwise Sequence Alignment
Translation of RNA to protein sequences	EMBOSS Transeq

When the sequence was correct, the plasmids for cell culture work were ready to use. For generation of transgenic animals the constructs were further processed as described in the Chapter 3.5.1.

3.3 Transient transfections and drug treatment in cell culture

3.3.1 Transfection of plasmids

The following plasmids were transfected into cell culture cells for different experiments:

plasmid	purpose
pIRES2DsRed2	Control transfection as a reference for the following three plasmids
Atf4-pIRES2DsRed2	Constitutive over-expression of ATF4
Atf6-pIRES2DsRed2	Constitutive over-expression of p50Atf6
Xbp1-pIRES2DsRed2	Constitutive over-expression of XBP1(s)
pEgfp-N1	Control transfection as an additional reference and (due to a strong EGFP-expression) a tool to estimate the transfection efficiency

Plasmids were transfected mainly into MLE12 cells and for some experiments MLE15 and MEL188-cells. The transfection was done with Lipofectamine2000 (invitrogen) on 12-well and 6-well plates, generally according to standard conditions of the manual, but with some adjustments.

Optimized transfection conditions:

1.) DNA-Lipofectamine complexes were incubated for 40 min before putting on the cells
2.) MLE12 and MLE15 cells were transfected in a transfection medium containing of 50 % culture medium and 50 % Optimem.
3.) 4-6 h after transfection the transfection mix was replaced by fresh culture medium

To estimate the transfection efficiency, cells transfected with pEGFP-N1 were observed under a fluorescence microscope with UV light a filter for green fluorescence and normal light respectively. The expression of the marker dsRed2 was to week and irregular for this purpose.
Transfected cells were used to check for transgene expression, downstream signalling, cell death and cell growth.

3.3.2 SiRNA-transfection

MLE12-cells were transfected with siRNAs against Atf4, Atf6 (final concentration of siRNA: 0.1µM). The transfection was performed with Darmafect 1 reagent (Dharmacon) according to the instructions. First two siRNAs against each gene (out of four delivered) were chosen considering their individual knockdown efficiency and specificity (not shown, sequences in 2.9). Then a combination of the "best" two against each gene was used. The total amount of siRNA per transfection was not altered. Therefore a siAtf4/Atf6 transfection contains only half the siRNA against Atf4 and Atf6 respectively like the single transfection. SiRNA transfected cells were generally harvested 48h after transfection.

3.3.3 Treatment with Thapsigargin (TG) and Brefeldin A (BFA)

1 µM TG (used as a 1 mM Stock in 100 % ethanol) and 10 µg/ml BFA (Stock: 10 mg/ml in DMSO) were used for chemical induction of ER-stress. The appropriate amount of the reagents was added with a micropipette into the cell culture medium. The plates were shaken by hand and put back into the CO_2-incubator.

3.4 Monitoring growth of MLE12

To monitor the effects of Atf4, Atf6 and Xbp1, cells transfected with those transgenes (and pIRES2dsRed2, pEGFP-N1 and WT-cells as controls) were counted on the culture dish at different time points. In detail, three independent transfections for each transgene were performed. Then one tenth of each transfection was transferred to a new well (6-well) with fresh culture medium. Fife spots for counting were labelled on the lid of the dish in a circle of 1 cm diameter around the middle of each well. For counting the middle of a microscopic light field (camera magnification: 12 x, Objective 20 x) was centred over a label, and adjusted for a clear picture. All cells in the digital images, that appeared to be adherent, were counted and the average of the fife countings was calculated. The same spots were photographed and counted after ON incubation (=0) and 9, 25 and 30 h later. Mean cell numbers and standard deviations were depicted in a logarithmic growth curve. The slopes of the three independent regression lines were used for statistics (t-test).

3.5 Transgenic mice

The vectors Atf4-PBI-L, Atf6-PBI-L, Xbp1-pBI-L and Chop-pBI-L were cloned as described above. For generation of transgenic animals they had to be further processed as described later in this chapter. The finalized constructs were then sent to EMBLEM (Monterotondo, Italy) for pronuclear injection and delivery of transgenic founder animals which were screened for transgene integration as described above. The offspring were then sent to CRL (Kisslegg, Germany) for breeding according to my instructions. For experiments the mice were sent to our facility.

3.5.1 Finalization of the constructs

To enable genomic integration of the constructs after oozyte injection, they were linearized. Furthermore the plasmid sequences of bacterial origin were removed to exclude all possible influences of these sequences. This was achieved by cutting with one or two restriction endonucleases which were chosen to cut out most of the amp resistance cassette and the *E. coli* ORI but not the other parts of the plasmid or the insert. The sites were identified with the aid of the program “NEB-cutter” which is public available in the internet.

vector	restriction endonucleases	reaction conditions
Atf4-pBI-L	*Bsr*BI	32 µl ATF4-pBI-L, 7 µl *Bsr*BI, 10 µl NEB2 buffer, 37 °C ON and one additional h with + 2 µl enzyme, reaction volume 100 µl
Atf6-pBI-L	*Bsr*BI	32 µl ATF6-pBI-L, 7 µl *Bsr*BI, 10 µl NEB2 buffer, 37 °C ON and one additional h with + 2 µl enzyme, reaction volume 100 µl
Xbp1- pBI-L	*Aat*II and *Drd*I	32 µl ATF6-pBI-L, 2 µl *Aat*II and 6 µl *Drd*I, 10 µl NEB4 buffer, 37 °C ON and one additional h with + 2 µl of both enzymes, reaction volume 120 µl
Chop- pBI-L	*Aat*II and *Ase*I	30 µl Chop- pBI-L, 4 µl *Aat*II, 10 µl NEB4 buffer, 37 °C ON and two additional h with + 2 µl enzyme, reaction volume 100 µl then PCR-purification and 230 µl digested plasmid, 4 µl *Ase*I 30 µl NEB3 buffer, 37 °C ON and two additional h with + 2 µl enzyme, reaction volume 300 µl

The largest fragment was isolated by gel purification. Gel purification is described in the “general methods” part. To keep up the required purity for oozyte injection, the electrophoresis-chamber was cleaned carefully and filled with fresh buffer. Furthermore the construct for the injection must not contain any Ethidiumbromid (ETBr). Therefore ETBr-free gel purification was performed. To detect the position of the desired fragment the sides of the gel were cut of and stained with ETBr. On a UV-table the bands were detected and labelled by a knife cut. Then the ETBr-stained slices were packed in disposal bags and placed next to the unstained part of the gel. The region with the biggest fragment (containing the transgene) was cut out. Then the purification proceeded as described.

To ensure that the fragment was sterile, particle free and dissolved in injection buffer at the appropriate concentration of 50 ng/µl, additional procedures were performed:

The eluate from gel-purification was diluted in 1 ml water. A 0.22 µm (PES) filter was washed with 1 ml water. Then the fragment was filtrated through this (now particle-free) filter into a 50 ml Falcon. Material remaining in the filter was washed into the filtrate with one additional ml water. The DNA was precipitated, washed

and air-dried. Finally it was resuspended in 200 µl injection buffer and the concentration was adjusted to 50 ng/µl (with injection buffer). The quality of the finalized construct was controlled on an agarose gel. At least 15 µg of each final construct were sent for oocyte injection.

3.5.2 From the construct to animal experiments

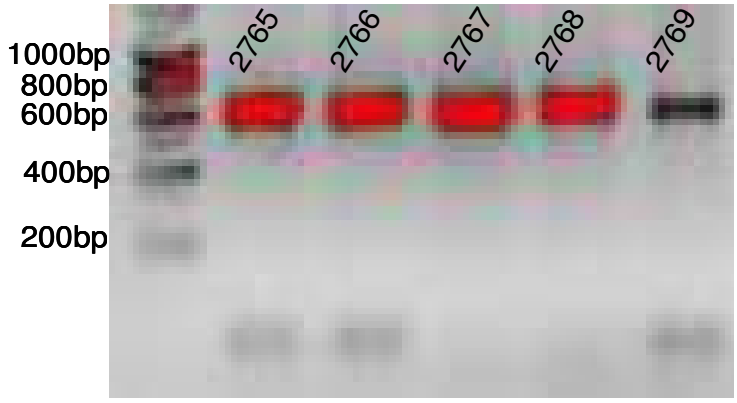
As mentioned above, the constructs were sent to EMBLEM for oocyte injection. EMBLEM provided isolated DNA of the potential founders for screening. New founders were screened with the PCR-procedures described in the “general methods” part. Specifically, PCRs against the luciferase part (usually with both described polymerase systems) and the insert part were performed, to verify the integration of the full length construct. The animals were then kept at CRL laboratory for breeding of double heterozygous (+-/+-), homozygous (++) and double homozygous (++/++) animals (explained in more detail below). All steps of the breeding program were performed according to my direct instructions.
The screening of the offspring for transgene-presence was generally a screening against the luciferase region of the construct (see chapter 3.1.20). The majority of the transgene integration screenings and screenings for SP-C-rtTA presence (the transactivator), including the preparation of genomic DNA, were kindly performed by Silke Händel.
++ mice were selected with qPCR, following the general method described above and further explained in the following chapter. Specifically the primer pair Hmbs-E4-forward and Hmbs-E4-reverse was utilized for the reference qPCR. Transgene homozygousity was investigated with the primers Luc-RT-PB-forward and Luc-RT-PB-reverse and SP-C-rtTA with the same primers also used for the general screening. These results were then verified by back breeding with WT animals.

3.5.3 The breeding program

The breeding of the transgenic animals had different aims, firstly the expansion of the respective line. It was desired to get more than one stable line for each transgene to enable the investigation of the effects of different integration sites. Secondly ++ lines should be generated to get a basis in which different transactivators can be bread in later on. Thirdly ++/++ mice (with the respective transgene and SP-C-rtTA as transactivator) should be generated for experiments.

And finally ++/++ lines were required for experiments and to get stable lines for later use.

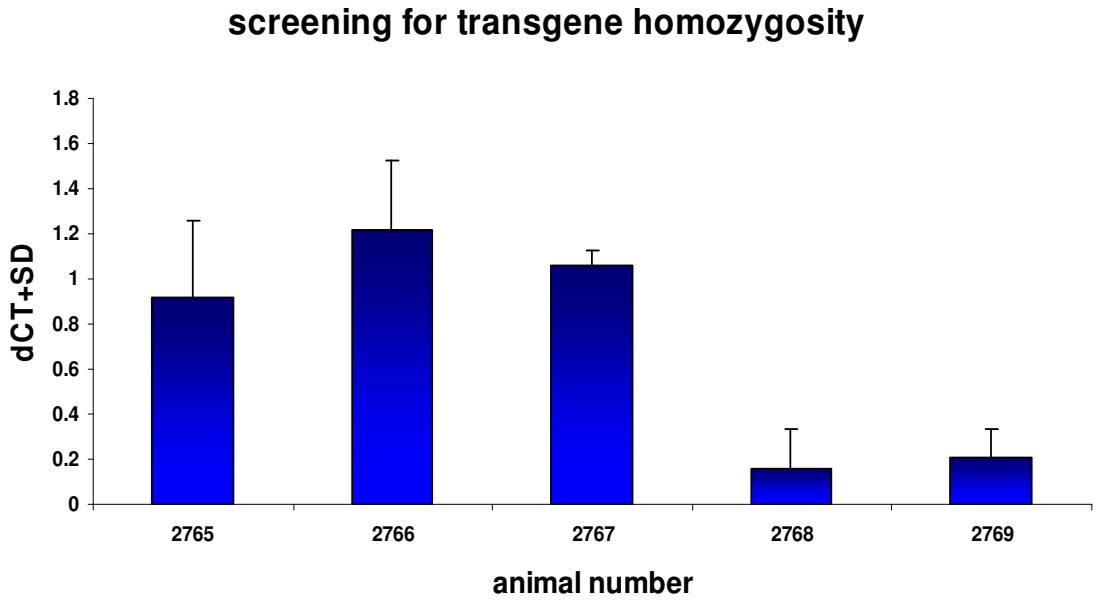
Therefore two different data had to be obtained separately for the transgene(s) and the transactivator: Is the mouse positive or negative for the construct and if it is positive, is it also ++? The first question was addressed by a conventional PCR-screening against SP-C-rtTA for the transactivator and against luciferase for all the transgenes. When homozygousity was possible the positive animals had to be further screened. As long as the integration site of the transgene is not known, only back breeding of the animals with wild type and subsequent screening of the offspring can prove homozygousity. If a larger number of pups is transgene-positive and none are transgene negative the parent is probably ++ as given by Mendel's law. However, statistically the majority of offspring from a +- breeding is not ++. Even if the negative animals are sorted out by conventional PCR-screening, the back breeding of the remaining (2/3) would require enormous cage capacities and unnecessary killing of many negative and heterozygous animals which could not be utilized. Therefore an additional qPCR based screening for homozygousity was established to select ++ animals. Only the animals identified as ++ by this method were than subject to an additional back-breeding and only those confirmed were regarded as truly ++. The qPCR was designed to differentiate between one and two copies (or 2 and 4 copies, in cases with tandem integrations of the transgene, which are also possible) of luciferase or SP-C-rtTA in the mouse genome which should lead to a CT-difference of one. The pre-selection was good enough to reduce the non- ++ animals in the back breeding to 1/3 (estimation).



A

***Figure 5*: Screening of transgenic animals (continued).**

***Figure 5* continue:**



B

C

	Preliminary Genotyp (qPCR screening)	Number of offspring from backbreeding	transgene positive offspring from backbreeding	confirmed genotype
2765	´++?	not successful	-	not confirmed
2766	´++	6	6	´++
2767	´++	11	11	´++
2768	´+-	no backbreeding	-	not confirmed
2769	´+-	no backbreeding	-	not confirmed

***Figure 5*: Screening of transgenic animals.**

The scheme depicts the process of a homozygousity-screening for a group of offspring from heterozygous (+-) Xbp1 animals. The method is independent of the inserted transgene. A) A conventional PCR-screening against luciferase (which is part of the constructs for all my transgenic mouse lines) revealed that all fife animals were positive for the transgene band at 600bp (see chapter 3.1.20 for the method). B) Therefore they were all included in the next step, the screening via qPCR (see Chapter 3.1.21). As the signal for No. 2765-2767 was about one dCT higher than for 2768 and 2769 the first three animals were preliminary categorised as homozygous (´++ or ´++? where lower dCT and a quite high SD of the three replicates left more doubts) and C) subjected to backbreeding. The animals 2766 and 2767 had offspring and as they were all transgene positive (the conventional screening to confirm that is the same method as depicted above and not shown here again), animals 2766 and 2767 were categorised as “confirmed homozygous (´++).

3.5.4 *In vivo* experiments, sampling and readout parameters

Available animals from breeding were sent to our facility for experiments. Animal experiments were authorized by “Regierungspräsidium Gießen” (V54-19c20-15(1)Gi20/10 Nr. 12/2009).
I generally set up experimental groups of five Dox-induced + five non-induced mice. These groups were littermate and gender matched. Furthermore the average weight of the mice at the beginning of the experiment was as equal as possible. The mice were taken into the experiment as young as possible, so they were usually 6-10 weeks old. The following table is an overview of the diverse experimental groups:

Table 1: **Experimental animal groups**

line	genotype	time of induction
ATF4	+-/+-	4 weeks
ATF4	+-/+-	16 weeks
ATF4	+-/+-	32 weeks
ATF4	++/++	4 weeks
ATF4	++/++	32 weeks
ATF6	+-/+-	4 weeks
ATF6	+-/+-	16 weeks
ATF6	+-/+-	32 weeks
ATF6	++/++	4 weeks
XBP1	+-/+-	4 weeks
XBP1	+-/+-	8 weeks
XBP1	+-/+-	32 weeks

Experimental groups were containing different lines (as only one line for each transgene was subject to experiments yet, only the name of the inserted transgene is listed here) of different genotypes (either +-/+- indicating animals heterozygous for transgene and transactivator or ++/++, which stands for double homozygous animals) and different times of transgene induction (4, 8, 16 of 32 weeks). With few exceptions (listed in the *supplementary tables 1-6*) the groups consisted of fife non-induced and fife Dox- fed animals. For details about the composition of the groups please refer to the text of this chapter.

Dr. Ingrid Henneke was kindly taking care of the experiments with the living animals and the sampling. She provided the raw data on death of animals, lung compliance, running time in the treadmill, body weight, the cell differentiation,

partly of the cell numbers in the BAL and the IVIS-signal intensity, as well as isolation and preparation lungs and other organs, H&E (Haematoxylin & Eosin) and Trichrome stained lung histological slides and the paraffin embedded slides for Immunohistochemistry. Which parameters were assessed from which samples can be taken from the processed data in the *supplementary tables 2-6*.

3.6 Microarrays

Microarrays based on Agilent technology were performed under supervision of Dr. Jochen Wilhelm with samples I provided. The general statistical analysis including some general readouts Gene Set Enrichment Analysis (GSEA) and preparation of diverse graphics (heat maps and correlation charts), following a design which was developed together, was also performed by Dr. Jochen Wilhelm. The Exon-arrays were performed by Dr. Alexander Sigrüner (*Uniklinikum und Institut für klinische Chemie*, Regensburg) and the general analysis was again undertaken under supervision of Dr. Jochen Wilhelm.

3.6.1 Overview

Four different experiments based on microarray technology were included in this thesis.
The first experiment was the gene regulation of MLE12 cells as downstream effect of Atf4, Atf6 and Xbp1(s). For this purpose MLE12 cells were transfected as described in the “General methods” chapter and the RNA was harvested after different times (22 h, 44 h and 66 h after transfection). The vector pIRES2dsRed2 served as a transfection-control (the reference against which the gene expression changes were calculated). As a positive control for strong ER-stress, cells transfected with this vector were treated with 1 µM TG 22 h after transfection and harvested 22 h later. This experiment was done with n=6.
A second experiment was designed to assess the gene regulation induced by early ER-stress. ER-stress was induced by 1 µM TG and the RNA was harvested after 0.5, 1 and 6 h (n=4 for treated cells, n=12 for controls). Only for the purpose of correlation analysis the two first microarray results were analyzed together. The gene expression changes and pathway analysis were done separately.
Thirdly, the effect of the transgenes (Atf4, Atf6, Xbp1 and pIRES2dsRed2 as reference, n=3) on miRNA expression was assessed by miRNA-arrays. For that purpose the cells were transfected with the respective plasmids and the RNA

containing miRNAs was isolated after 24 h (as described in the general methods part).
In a last microarray-experiment the effects of equal transfections as for the miRNA experiments (n=3) on mRNA splicing were assessed. Enriched mRNAs from the mentioned transfections (“first experiment”) were used for 400K exon-arrays (pooled, n=3).

3.6.2 Technical details

MRNA-arrays:

Purified total RNA was amplified and Cy-labeled using the dual-color QuickAmp labeling kit (Agilent, P/N 5190-0444) following the kit instructions. Per reaction, 1 µg of total RNA was used. The samples were labeled with either Cy3 or Cy5 to match a balanced dye-swap design. Cy3- and Cy5-labeled aRNAs were hybridized ON to 4 x 44K 60mer oligonucleotide spotted microarray slides (Mouse Whole Genome 4 x 44K; Agilent Technologies, P/N G4122F). Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol. The dried slides were scanned using the GenePix 4100A scanner (Axon Instruments, Downingtown, PA). Image analysis was performed with GenePix Pro 5.1 software and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software and the limma package from BioConductor. The intensity values were quantile normalized before averaging. Genes were ranked for differential expression using a moderated t-statistic. Candidate lists were created by adjusting the false-discovery rate to 5 %. Pathway analyses were done using the function geneSetTest from limma on the ranks of the t values.

MiRNA arrays

Purified total RNA was amplified, labeled and hybridized using the miRNA Complete Labeling and Hyb Kit (Agilent, P/N 5190-0456) following the kit instructions. Per reaction, 200 ng of total RNA was used. The Cy5-labeled aRNA were hybridized ON to 8 x 11K 60mer oligonucleotide spotted microarray slides (Mouse miRNA Microarray Release 15.0, 8 x 15K, P/N G4471A-029152). Hybridization and subsequent washing and drying of the slides were performed following the Agilent hybridization protocol. The dried slides were scanned using the GenePix 4100A scanner (Axon Instruments, Downingtown, PA). Image

analysis was performed with GenePix Pro 5.1 software, and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using the R software and the limma package from BioConductor. The intensity values were quantile normalized before averaging. Genes were ranked for differential expression using a moderated t-statistic.

Exon-array-analysis

400k exon arrays (Affymetrix) were performed by Dr. Alexander Sigrüner (Regensburg). The basis for the data analysis by Dr. Jochen Wilhelm was the column "gProcessedSignal" of the provided text files, containing the results of the image analysis of the scanned slides. The Evaluation was done in R, version 2.12.2 (R Development Core Team 2011) with the package "limma" (Smyth 2005). The annotation of the spots followed the attached gene annotation list. The intensity-values were normalized in quantiles and subject to logarithmic transformation before further use. For the analysis of the (mean) differential expression (DE) of the genes, the means of all probes belonging to a gene were calculated. The calculation of the DE was done by a linear model, using the function "lmFit" for the contrasts "Atf4-dsRed", "Atf6-dsRed" and "Xbp1-dsRed". The calculation of the test-statistics for H0: DE=0 was done by the function "eBayes" about moderated t-statistics. Selection of DE exons was performed with a false discovery rate (FDR) of 5 % (Benjamini 1995).

3.7 Statistics

Statistics (with the exception for the statistics of the microarrays which were performed by Dr. Jochen Wilhelm and are discussed in 3.6.2) were performed as a heteroscedastic t-test by the Microsoft Excel function “ttest”. T-tests were generally performed as a two-directional test. Knockdown and over-expression were tested by unidirectional t-tests. In “Results” and “Discussion” p-values <0.05 are called “significant”. This has to be understood as “assuming normal distribution of the samples, these results were significant”. The p-values were not corrected for multiple testing. Statistics were generally performed for “independent” experiments, but the term can stand for different degrees of independence.
The RNAs for the study of single Atf4, Atf6 and Xbp1 over-expression were generated by transfection of three sets of cells (from the same passage number), independently thawed and put into culture in different weeks. At each of the

time points two “independent” pools were generated. The RNAs were also isolated in three steps. For other cell culture experiments, independent transfection means “transfection with separately mixed transfection mixes at the same time point”. In animal experiments the term stands for “data generated from different animals”.

4. Results

4.1 Verification of experimental tools

In the context of this chapter, experimental tools, procedures and animals which were “produced” *de novo* to enable the research are described. The question, in how far these “tools” are generally functional is also addressed. The headline was only chosen to make clear, that these results are “technical” results and do not bear new scientific information by themselves. The utilized mice are, of course, not objects but living animals, which have to be treated with respect.

4.1.1 Vectors for transfection *in vitro*

For the purpose of in vitro studies, plasmid vectors for over-expression of murine ATF4 (Uniprot Q06507, sequence according to the indicated amino acid (AA) 345, K→Q sequence conflict), p50ATF6-α (Uniprot Q8BZ84, AA23-389) and the spliced form of Xbp1 (Uniprot O35426, isoform 2), based on the plasmid pIRES2-DsRed2 (Clontech) depicted below were produced.

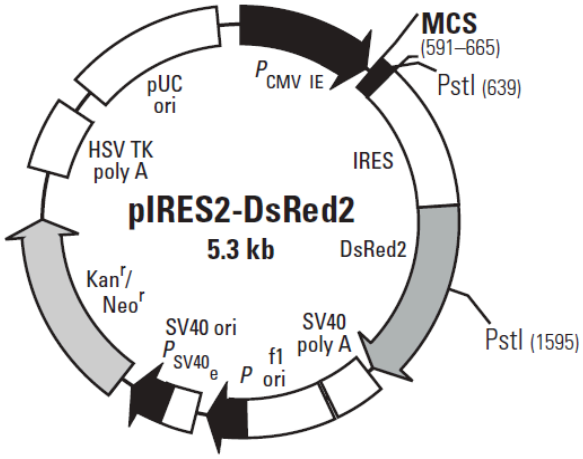


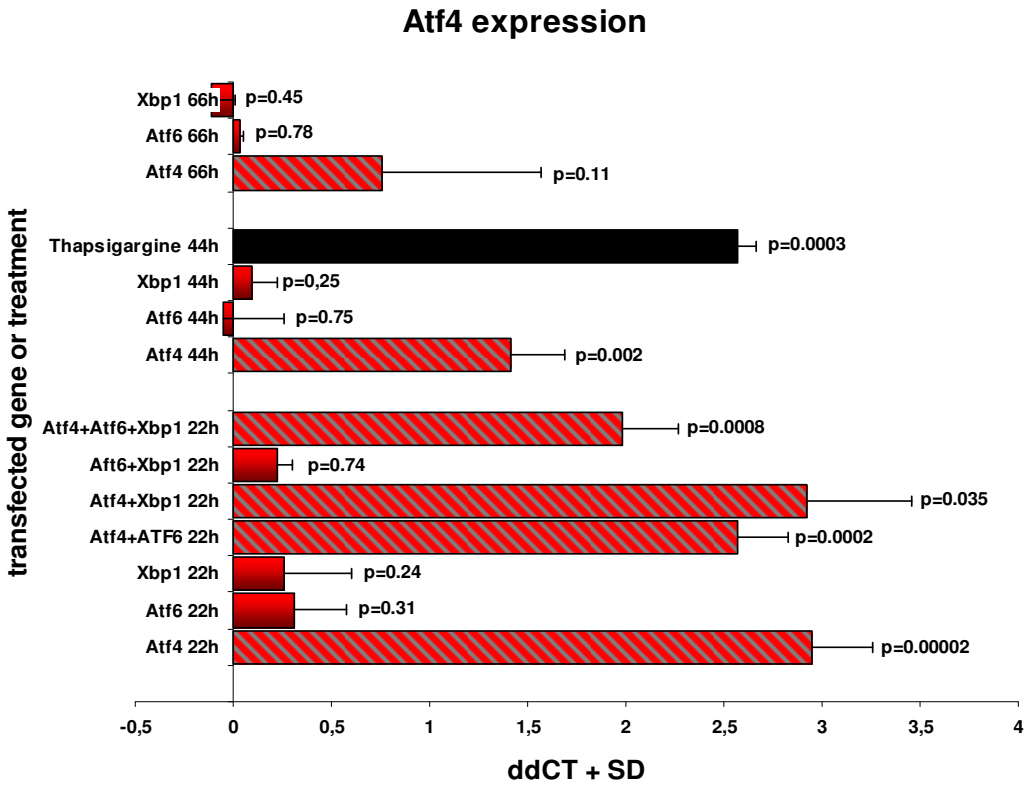
***Figure 6*: The vector used for cell culture experiments.** Each transgene sequence was inserted in to the multiple cloning site (MCS) of the depicted vector as described in detail in chapter 3.2. It is then transcribed together with the internal ribosomal entry site (IRES) and the fluorescent marker DsRed2 sequence and a SV40 poly A sequence on one cistron driven by the indicated CMV promoter. The translation of the transgene is then done as a transcript, separately from the DsRed2 (for which the IRES is necessary). The pUC origin of replication (ori) and the Kanamycin resistance cassette (driven by an SV40 promoter and tailed by HSV TK poly A) are required for propagation of the plasmid in *E. coli.* The f1 ori indicated, does not play a role for my applications. The vector **(continued)**

***Figure 6* (continue):** map was taken from the pIRES2-DsRed2-datasheet provided with the vector (Clontech)

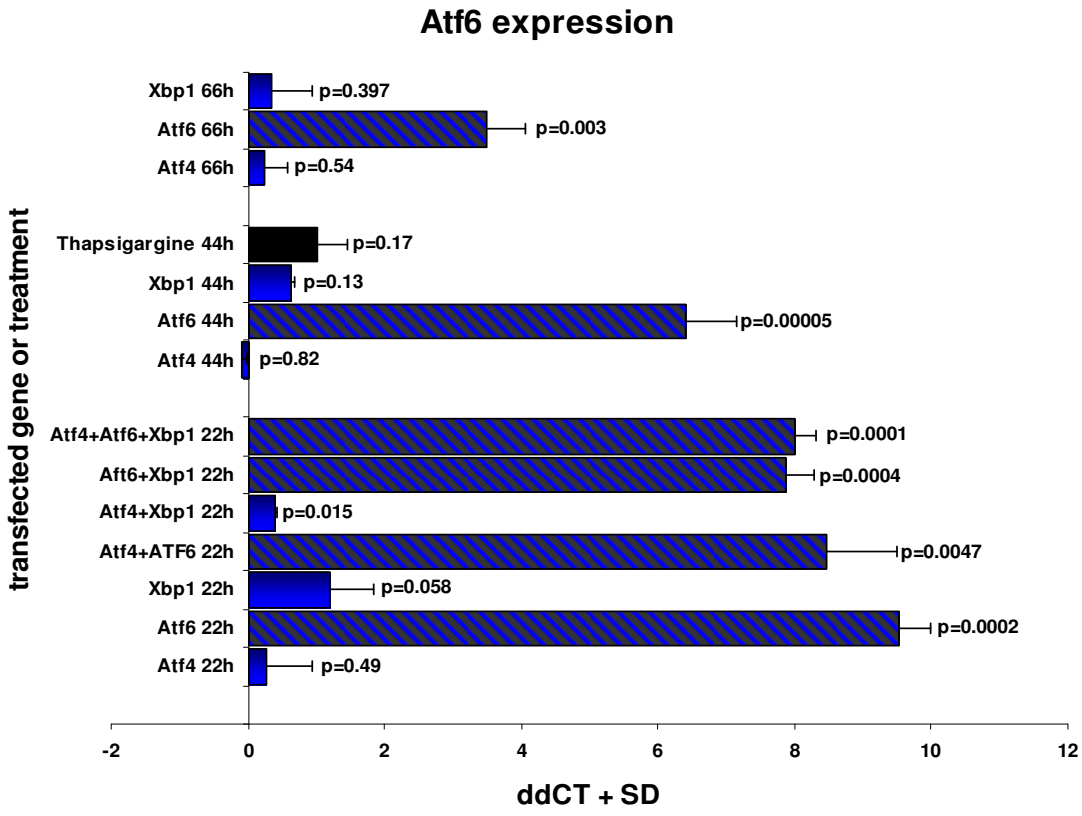
The bi-cistronic vector allows transcription, driven by the CMV-promoter, of the respective transgene and the fluorescent reporter dye dsRed2 on one cistron. The dye is not attached to the transgene, but expressed separately by an internal ribosomal entry site (IRES). A Kozak-sequence (Kozak 1987) was cloned in front of the start codons of the transgenes to enhance ribosome binding and thereby the translation of the protein.

ATF4, ATF6 and XBP1 were successfully over-expressed in MLE12 after transfection of the respective plasmid vector (see *figure 7*). Furthermore the transfection efficiency was estimated to range between 80-100 % as assessed by parallel transfection with a green fluorescent marker (pEGFP-N1, not shown). An induction of the ATF4 and XBP1-protein was clearly detected 24 h after transfection. ATF6 appeared to be reduced by transfection with the empty vector and initially also by the ATF6-vector. However, ATF6 accumulated when over-expressed and after 48 h its levels surpassed those observed in the untreated and empty-vector-controls (see *figure 7 D+E*)

On mRNA-level, expression levels were assessed at three different time points after transfection. 22 h after transfection, the expression of the transgenes was at its maximum (ddCT for Atf4: 2.94; for Atf6: 9.51; for Xbp1: 7.38). After 44 h the transgene-levels had declined but were still significantly increased as compared to the vector control (ddCT for Atf4: 1.41; for Atf6: 6.39; for Xbp1: 4.88). This tendency continued and 66 h after transfection, Atf4 was no longer significantly increased (ddCT for Atf4: 0.76; for Atf6: 3.49; for Xbp1: 2.28). Double and triple transfections with these factors were also effective, as assessed after 22 h. As a lesser amount of the individual plasmids was transfected in the combinations, the transgene expression-level was then also lower than in the single transfections (ddCT for Atf4: 1.98-2.92; for Atf6: 7.88-8.46; for Xbp1: 5.43-6.73) (see *figure 7 A-C*).

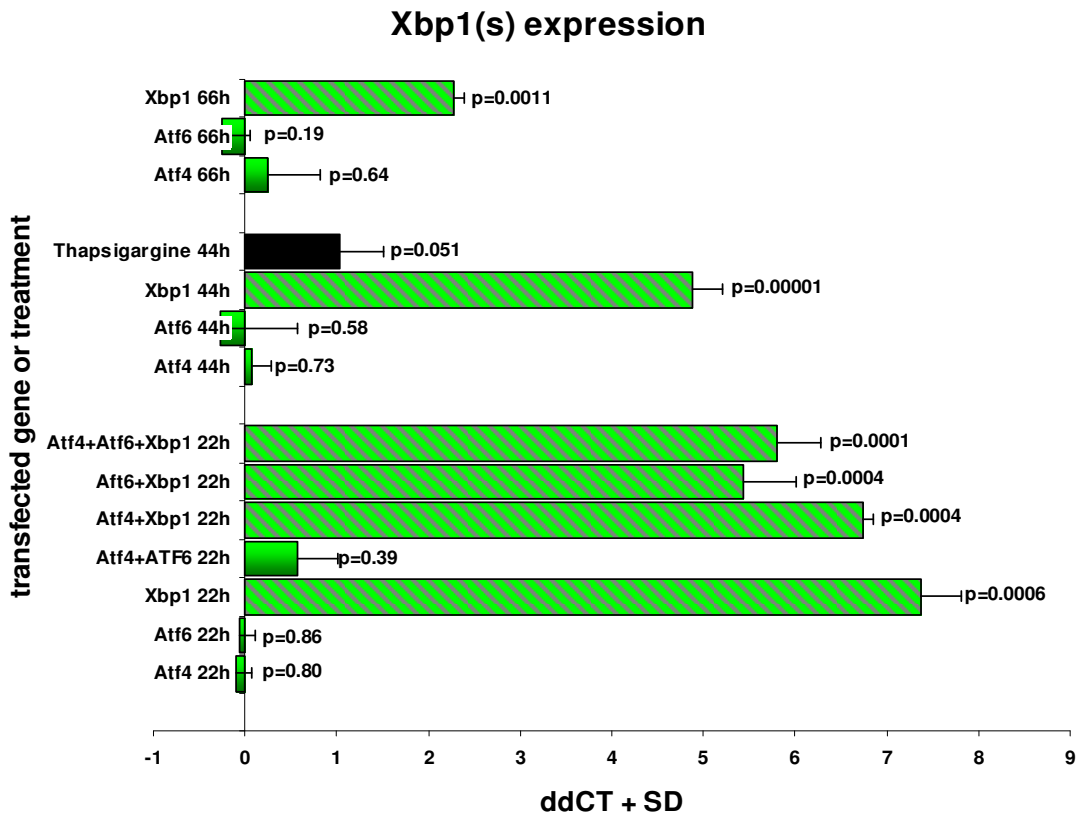


A

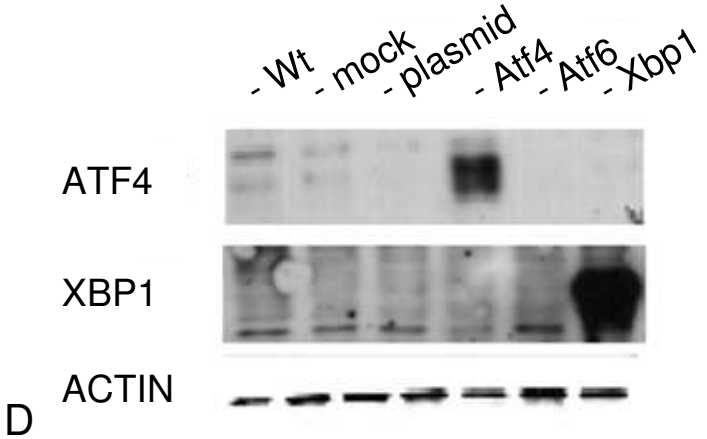


B

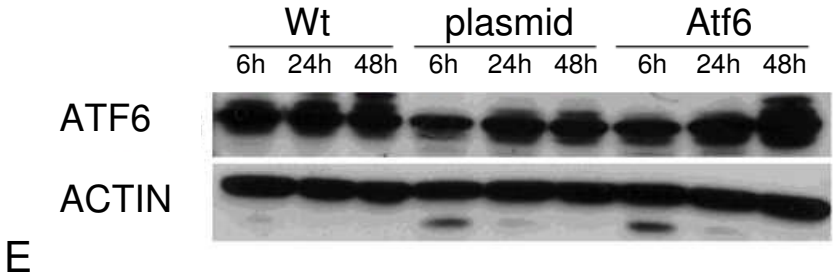
***Figure 7*: Expression of Atf4, Atf6 and Xbp1 in MLE12-cells (continued).**



C



D



E

***Figure 7*: Expression of Atf4, Atf6 and Xbp1 in MLE12-cells. (continued)**

***Figure 7*: Expression of Atf4, Atf6 and Xbp1 in MLE12-cells.** A-C) MLE12 cells were transfected with the cell culture constructs for Atf4, Atf6 or Xbp1(s) (see 4.1.1) for 22 h, 44 h or 66 h as indicated or with half the amount of a combination of two transfection mixes or one third of the three together (for 22 h). Transfections with the empty plasmid (pIRES2dsRed2) for the same durations served as a reference to calculate the ddCT. TG treatment (black bars) was done on top of 22 h empty vector transfection and another 22 h incubation, leading to the indicated 44 h. Red indicates a qPCR for Atf4, blue for Atf6 and green for Xbp1(s). Samples where the respective transgene was part of the transfection mix were highlighted with dashed bars (n=3). D+E) Protein expression of ATF4 and Xbp1 24 h after transfection (D) and ATF6 at the indicated time points. ATF4 and Xbp1 were exclusively detected when transfected and not in WT (untreated), mock (only transfection reagent without DNA) or transfection with the other UPR transcription factors. P50ATF6 was already present in WT cells and decreased upon transfection with the empty plasmid. However, transfection with the ATF6 plasmid even though primarily also leading to an ATF6-decrease as compared to WT, was leading to increased ATF6-levels at least after 48 h (earlier as compared to empty plasmid transfection). Actin served as a control for equal loading.

4.1.2 Vectors for generation of transgenic animals

For the generation of transgenic animals with conditional over-expression of murine ATF4 (Uniprot Q06507, sequence according to the indicated AA 345 K→Q sequence conflict), p50ATF6-α (Uniprot Q8BZ84 AA23-389) the spliced form of Xbp1 (Uniprot O35426, isoform 2), and CHOP (Uniprot P35639), vectors based on the plasmid pBI-L (Clontech) were cloned.

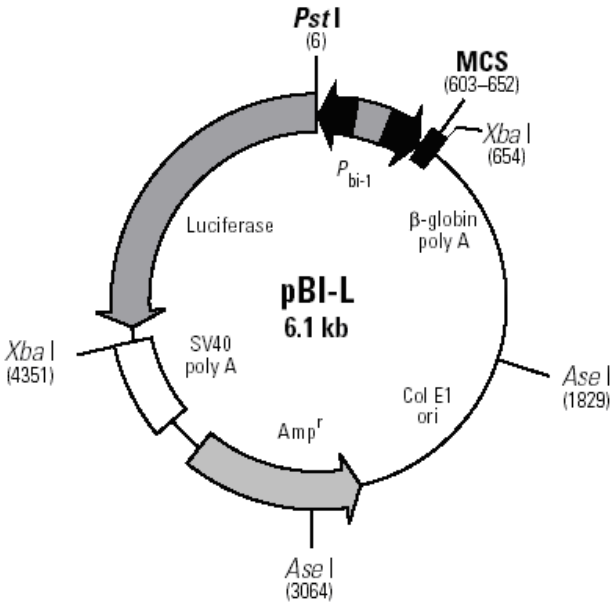


***Figure 8*: The vector used for oozyte injection.** The transgenes were cloned into the indicated multiple cloning site (MCS). The bidirectional **(continued)**

Continue *figure 8*: promoter (p_{bi-l}) consists of a minimum CMV promoter and repetitive tetracyclin- responsive elements (tetO7). It is inactive unless rtTA with bound doxycycline (Dox) (provided by other sources) binds to the tetO7. Also depicted are diverse sequences for polyadenylation (SV40 poly A and ß-globin poly A) propagation in *E. coli* (Col E1 ori) and selection during propagation (Amp^r= Ampicillin resistance cassette). Amp^r and Col E1 ori were cleaved out by digestion with restriction endonucleases before oozyte injection. The vector map was taken from the pBI-L-datasheet provided with the vector (Clontech).

The bidirectional vector allows inducible transcription of luciferase and the respective transgene on different cistrons, but driven by the same promoter. Activation of the promoter requires the presence of the transactivator rtTA and the binding of the antibiotic Dox to the rtTA. The transactivator can be added by transfection with an additional vector in cell culture systems or by crossbreeding with a transactivator line *in vivo*. The Dox can be added to the cell culture as a solvent and to the transgenic animals as an injection or (as in this work) by nutrition.

4.1.3 Confirmation of generation of transgenic mouse lines and current status of the breeding program

The vectors for the transgenic animals were linearized and send for oozyte injection as described in “material and methods” (see 3.5.1 and 3.5.2). The pups generated by this method were screened for the insertion of the transgenes as described in 3.1.20 and 3.5.3. Up to now, mouse lines with confirmed integration of the described constructs for ATF4, ATF6 and XBP1 over-expression were confirmed, whereas the oozyte injections for CHOP did not yet lead to success[3].
At the end of July 2011 the situation of the Atf4, Atf6 and Xbp1 lines was the following:
Lines from two Xbp1-founders existed. The first line was stable and some ++ males and females lived and were in breeding. However, the ++ situation was not yet stable and there was no transactivator breed in. Subsequently, no experiments have been performed with that line.
For the second Xbp1 line, experiments with a lot of +-/+- mice from backbreeding (see 3.5.3 for backbreeding and *table 1* for an overview of the experimental groups) of ++/++ mice were performed. After that the breeding success stopped and many mice died. Only three mice of the line survived and are all in breeding for colony expansion.

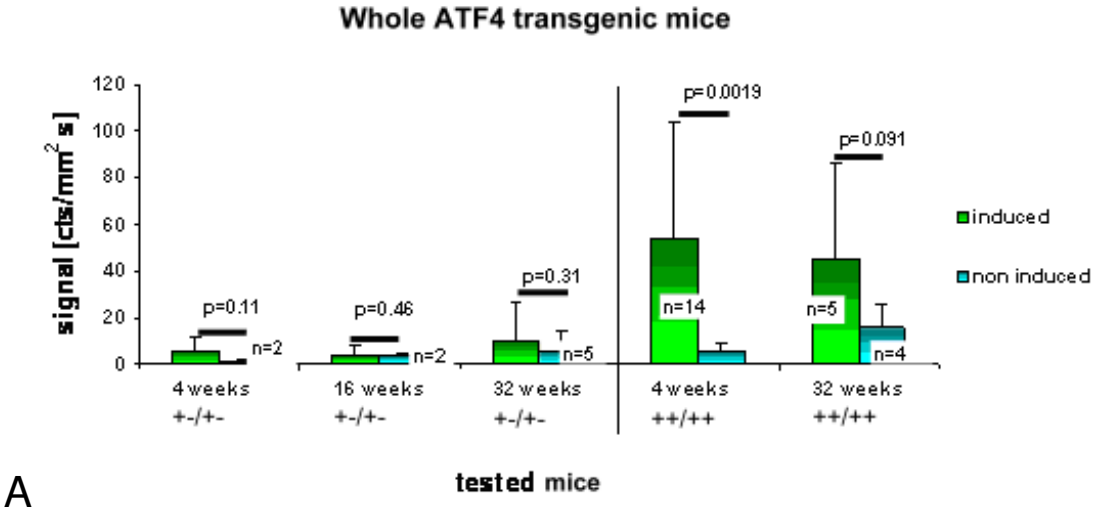
[3] One founder for the line was obtained lateron.

There was only one line for Atf6 available, for which ++ and ++/++ animals exist. Animals of this line were already used for experiments and a +-/+- (5+5) group and a smaller ++/++ (3+3) group was still in the experiment.
Atf4 was represented in two lines. The first one was stable ++/++. Animals from this line were used for experiments (see table 1). The second Atf4 line consisted of limited numbers of ++ and single or double +- animals. The line as such was stable but sufficient numbers of animals for experiments could not be achieved.
As a side product of the breeding of the first ATF4 line, a transgene-negative but transactivator ++ line was generated, which was breeding nicely and can be used to speed up the generation of double-positive mice.

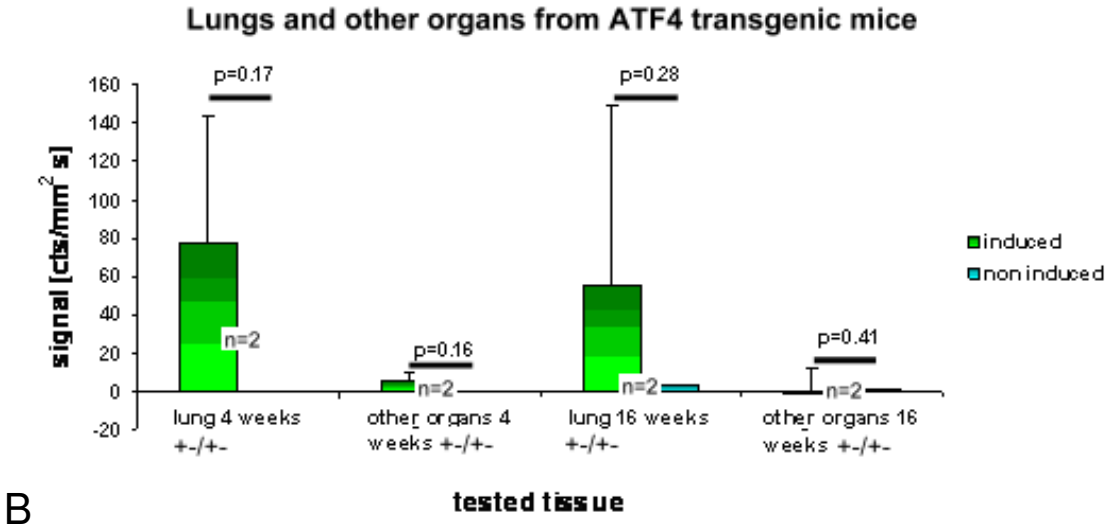
4.1.4 Transgene expression in conditional transgenic lines

Because of the current status of the breeding, the assessment of transgene expression of the Atf4, Atf6 and Xbp1 transgenic mouse lines is not yet fully completed. The existing lines of evidence for a successful generation are summarized here: The first line of evidence stems from the results of the *in vivo* imaging. The DNA-constructs for *in vivo* application are designed to express the respective transgene and the reporter luciferase as separate mRNAs, driven by the same bidirectional promoter. The level of luciferase expression should therefore correlate with the (mRNA) level of the transgene. *In vivo* detected luciferase-activity should therefore be an indirect line of evidence for a successful transgene insertion and activation. Only those organs, where the luciferase (and therefore the transgene as well) is expressed should emit light from a luciferin-luciferase-reaction. For convenience *in vivo* the signal from the whole mouse was quantified. When available, the signal from taken out organs was observed additionally to check for the location of the induction. For all the three observed Atf4, Atf6 and Xbp1-lines there was at least a tendency for a lung-specific luciferase-induction in +- mice. However, the effect size and level of significance was very different for the lines. The induction in Atf4-mice was poor and not significant. Atf6 mice had a significant, but low induction after 4 and 16 weeks. The light emission in the Xbp1-mice was by far the highest observed, however it was only significant in the 8 and 32-weeks induced groups.
In some individual induced ATF4 mouse-lungs quite strong signals could be detected but the effect was not significant. There was no strong signal from any other organs than the lung in these animals. ++ ATF4-mice were showing a much stronger and significant induction after 4 and 32 weeks. The same holds true for

the ++ 4-weeks Atf6-mouse group. Paradoxically most of the signal from these mice was gone after taking out the organs. The remaining signal however was lung specific and significant. In Xbp1-mice the strong signal observed in the whole mouse could be confirmed in the mouse lung, where the induction is also significant. In brain and thyroid of this line, a moderate uninduced signal could be observed (see *figure 9*).



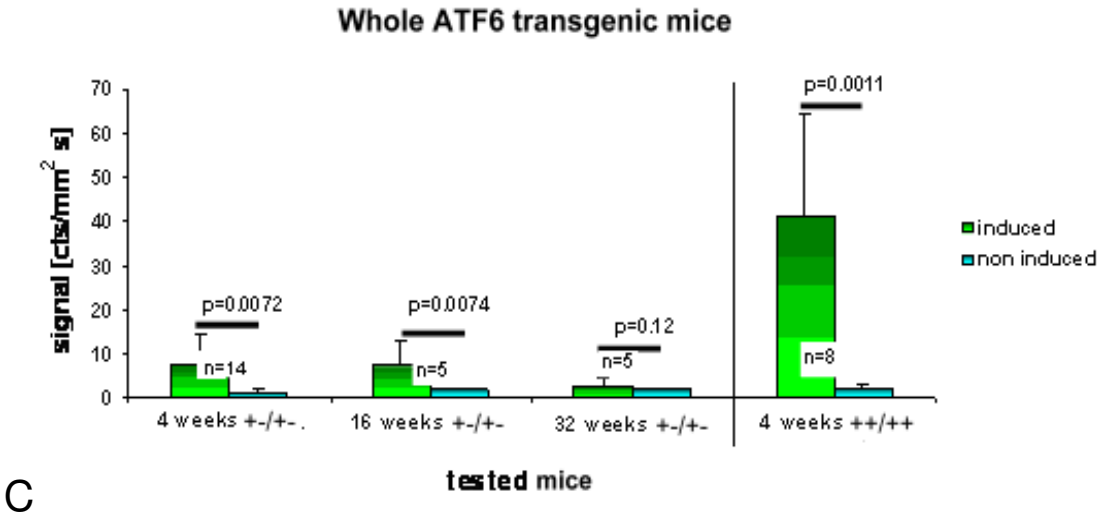
A



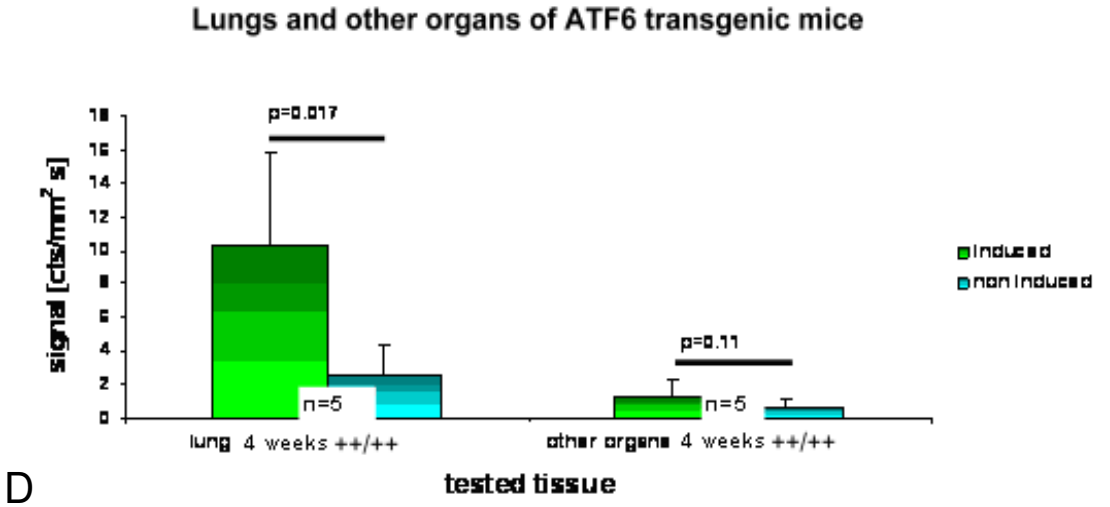
B

***Figure 9*: *In vivo* imaging of Atf4, Atf6 and Xbp1 transgenic mice employing the luciferase reporter gene (continued)**

Continue *figure 9*:



C



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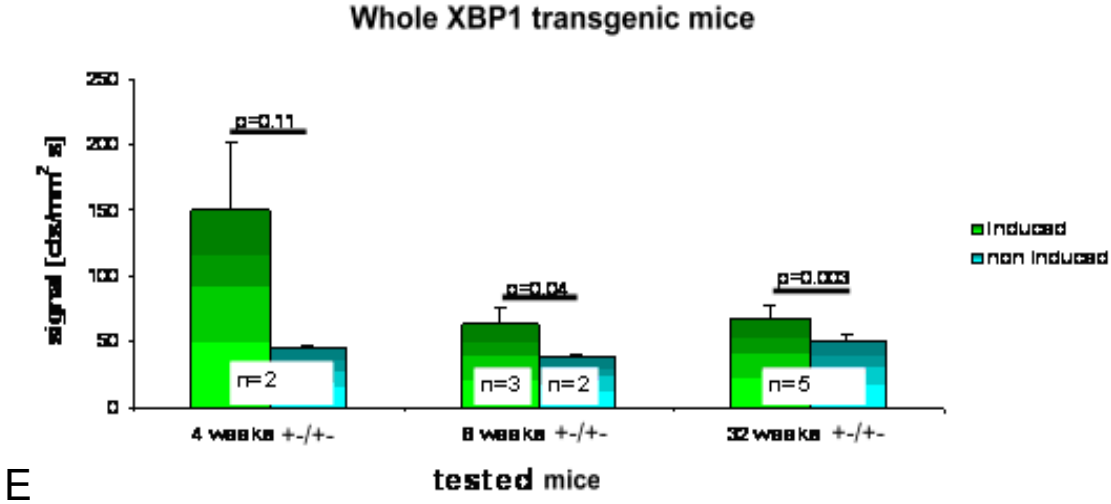
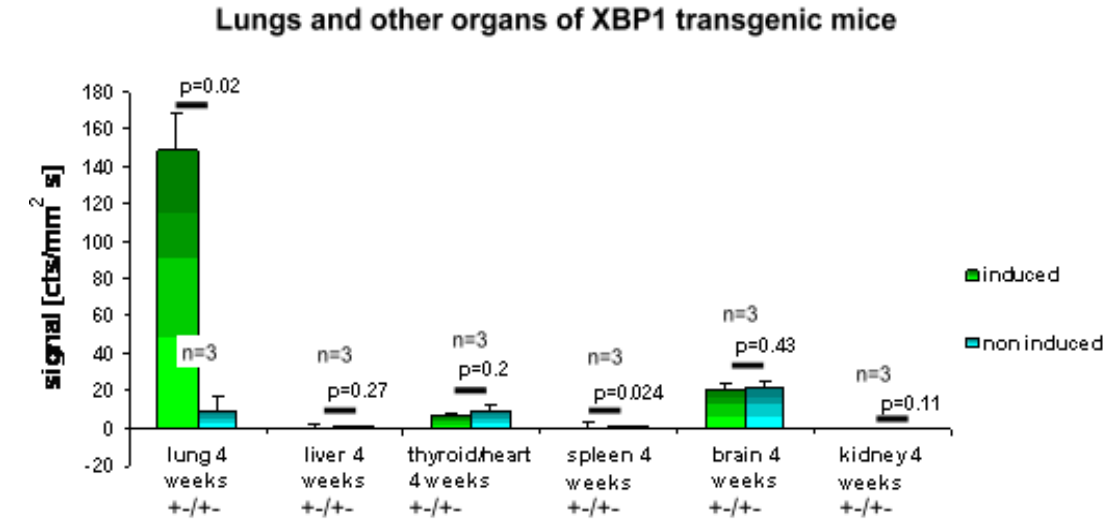


E

***Figure 9*: *In vivo* imaging of Atf4, Atf6 and Xbp1 transgenic mice employing the luciferase reporter gene (continued)**

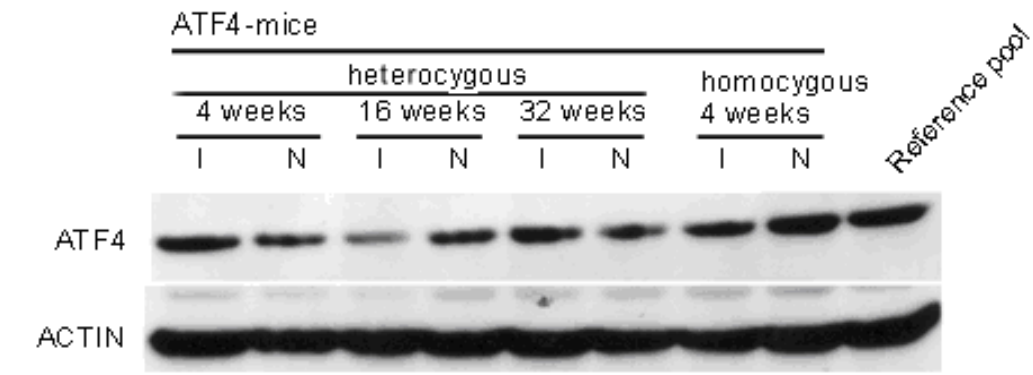
Continue *figure 9*:



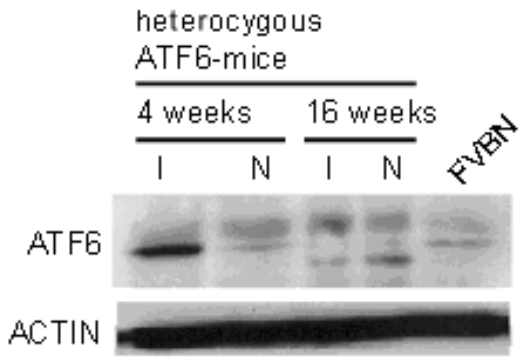
F

***Figure 9*: *In vivo* imaging of Atf4, Atf6 and Xbp1 transgenic mice employing the luciferase reporter gene.** The emitted light from mice was quantified as the signal of an equal rectangular frame around the mice and referenced to a reference field next to the mice (A, C, E). The organs were placed in a 6-well cell culture dish and quantified as the signal from the well (where no organ except the lung gave a signal, the values are expressed as a mean, containing the signal from for liver, thyroid / heart, spleen, brain and kidney) (B, D, F). All mice of a distinct genotype and, treatment (induced / non-induced) were summarized in one bar, when the IVIS-screening was performed at the indicated time point. +-/+- = heterocygous for transgene and transactivator; ++/++ = homocygous for transgene and transactivator.

The best indicator for successful induction of the transgene induction is the comparison of protein expression. For this purpose WBs were performed with lung homogenates, not with isolated AECII. For the investigated Atf4-line, a specific ATF4 band was detected. However, there was no sign for a robust increase in expression of ATF4 detectable as a result of Dox-feeding in +- or ++ animals (f*igure 10 A*). +-/+- Atf6-mice were showing increased levels of ATF6 for the four weeks group, but not for the 16 weeks group (see *figure 10 B*). This was shown in blots with pooled samples and confirmed in additional blots with single mouse samples (not shown). ++ ATF6 mice had an increased basic ATF6-level which could clearly be enhanced by Dox-nutrition (see *figure 10 C+D*). The Xbp1 antibodies tried, failed to give a clear specific signal in WB of lung homogenates (Data not shown).



A



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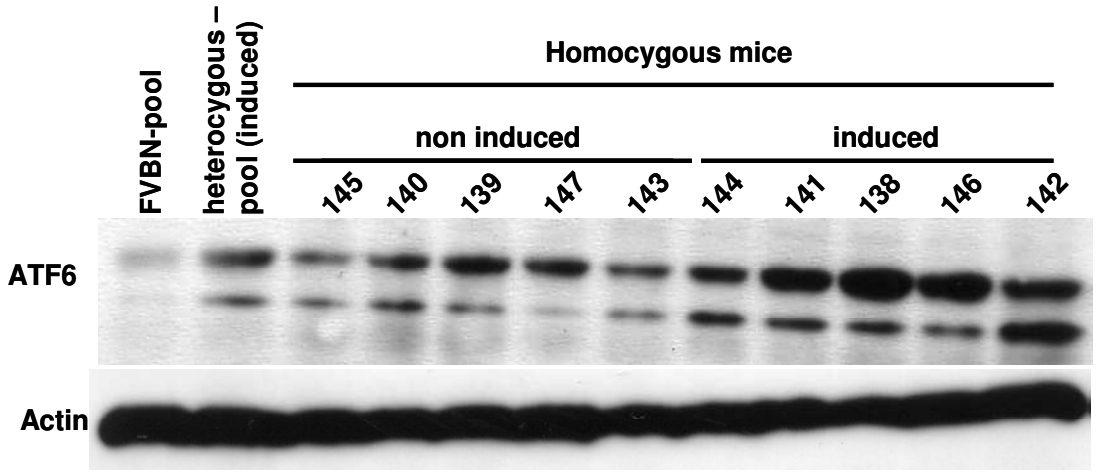
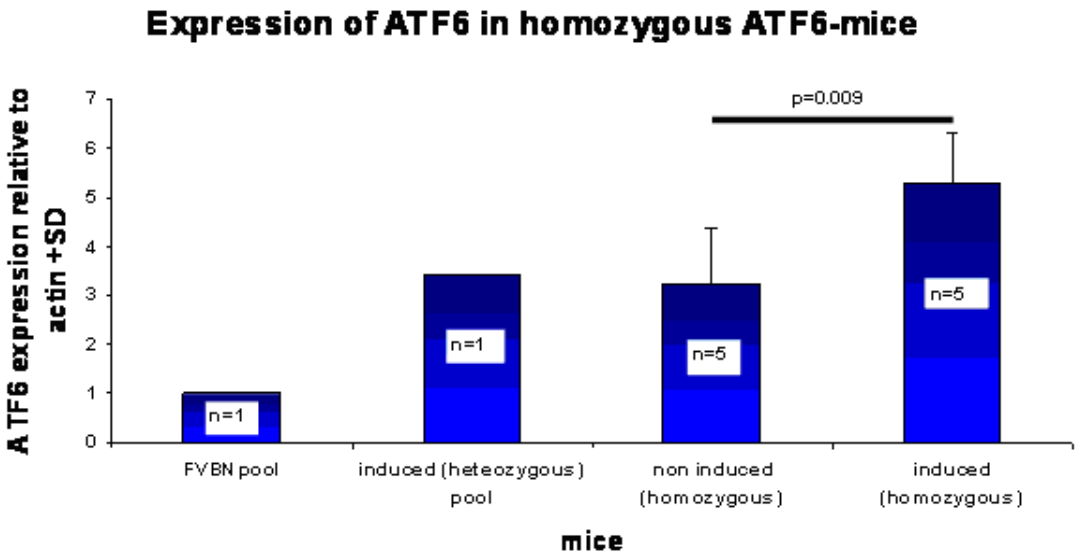


C

***Figure 10*: Western blots for transgene expression in heterozygous and homozygous ATF4 and ATF6-mice (continued).**

Continue *figure 10*:



D

***Figure 10*: Western blots for transgene expression in heterozygous and homozygous ATF4 and ATF6-mice.** A) WB for ATF4 in pooled lung homogenates of ATF4 mice after the indicated time in the experiment. I=induced, N=non-induced. The reference pool consists of a mixture of homogenates from non-induced Atf4, Atf6, Xbp1 and FVBN mice. B) WB for ATF6 in pooled lung homogenates of +- ATF6 mice after the indicated time in the experiment. I=induced, N=non-induced. The mixture of two FVBN-mouse lung homogenates serves as a control. C) WB for ATF6 in pooled lung homogenates of ++ ATF6 mice after four weeks in the experiment. A pool of two FVBN mouse homogenates and the pool of +- -mouse homogenates induced lung homogenates from B serve as controls. The numbers 138-147 are the ear tag numbers of the individual mice. D) Densitometric quantification of C.

Immunohistochemistry (IHC) is less reliable for quantitative analysis as compared to Western blots. But it allows a localization of the protein of interest within a certain cell type by staining of a cell specific marker in parallel sections (here: pro-SP-C, which stains alveolar type II cells). IHC-slides for ATF4 revealed a generally strong and not AECII-specific signal for ATF4. Therefore they did not allow conclusions about the induction of the transgene (Data not shown). A robust ATF6 signal was specifically detected in induced ATF6-mouse slides but not in uninduced mice or FVBN control slides. Parallel pro-SP-C staining confirms that AECII are the primarily affected cell population. Additionally, a population of bronchial epithelial cells expressed the transgene in induced but not in non-induced animals. Furthermore it could be shown that the ATF6-downstream

target BIP was highly expressed in AECII of induced ATF6-mice specifically, indicating functionality of the transgene.
Xbp1 IHC-slides revealed a similar expression pattern. An increased Xbp1 expression was detected in AECII and a population of bronchial epithelial cell of induced Xbp1 animals.

A) Controlmice – no transgene

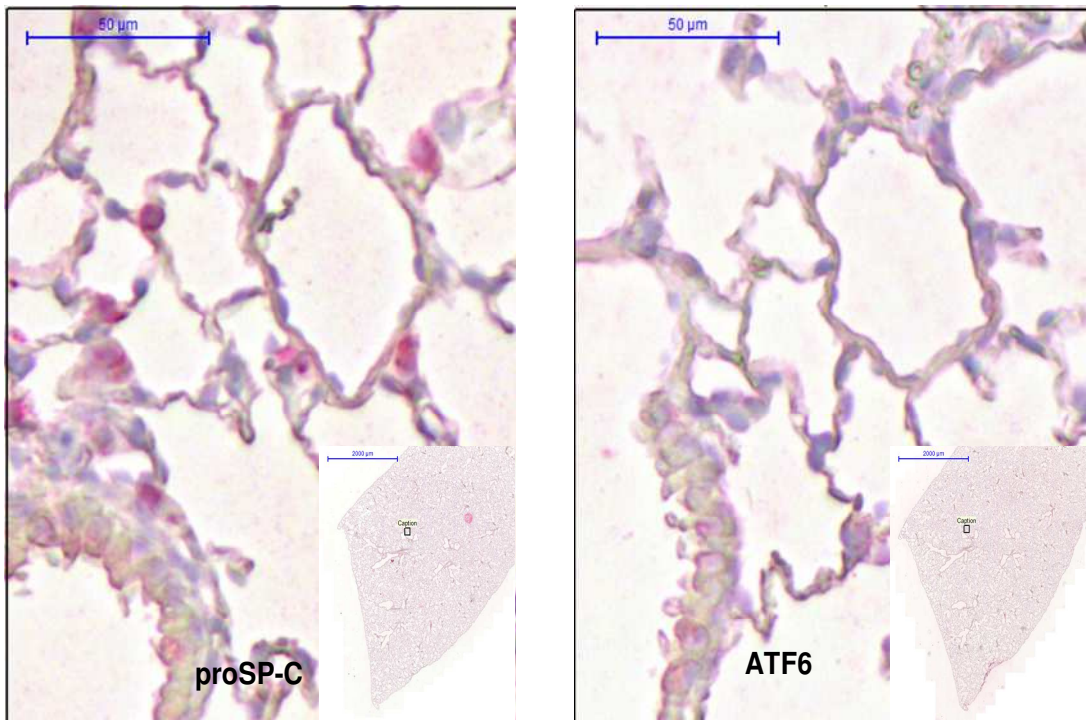
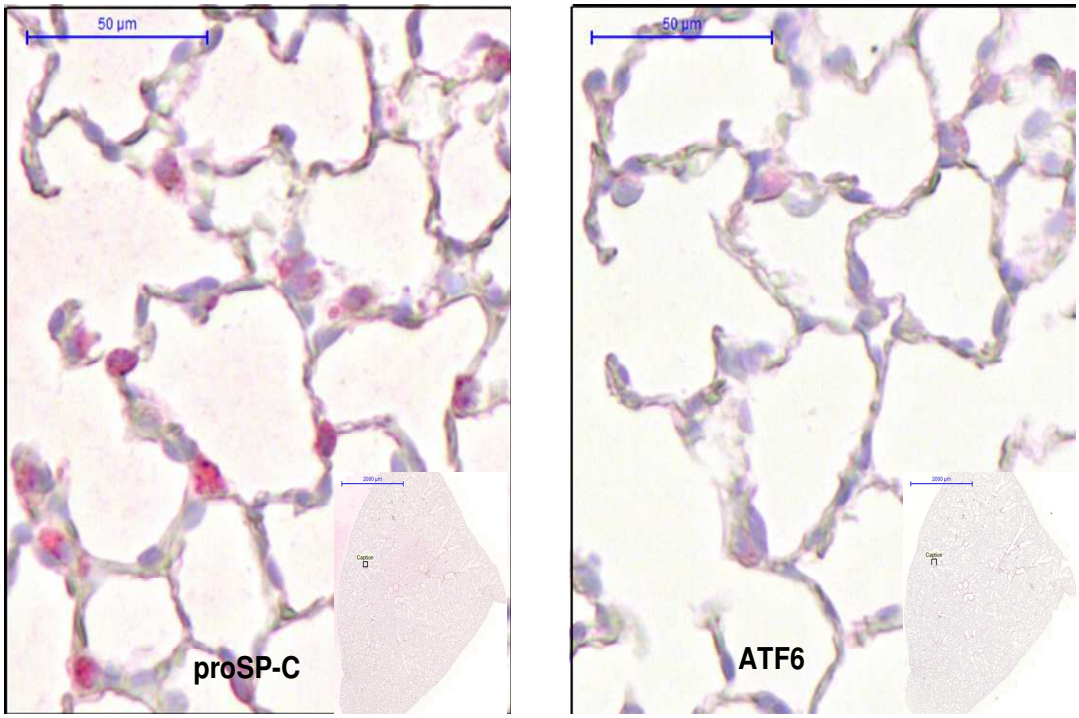


***Figure 11*: Immunohistochemistry of ATF6 (A-E) and Xbp1 (F-H)-mice (continued)**

Continue *figure 11*:

B) ATF6 transgenic mice,+-, no induction with doxycyclin



C) ATF6 transgenic mice,+-, 4 weeks on doxycyclin

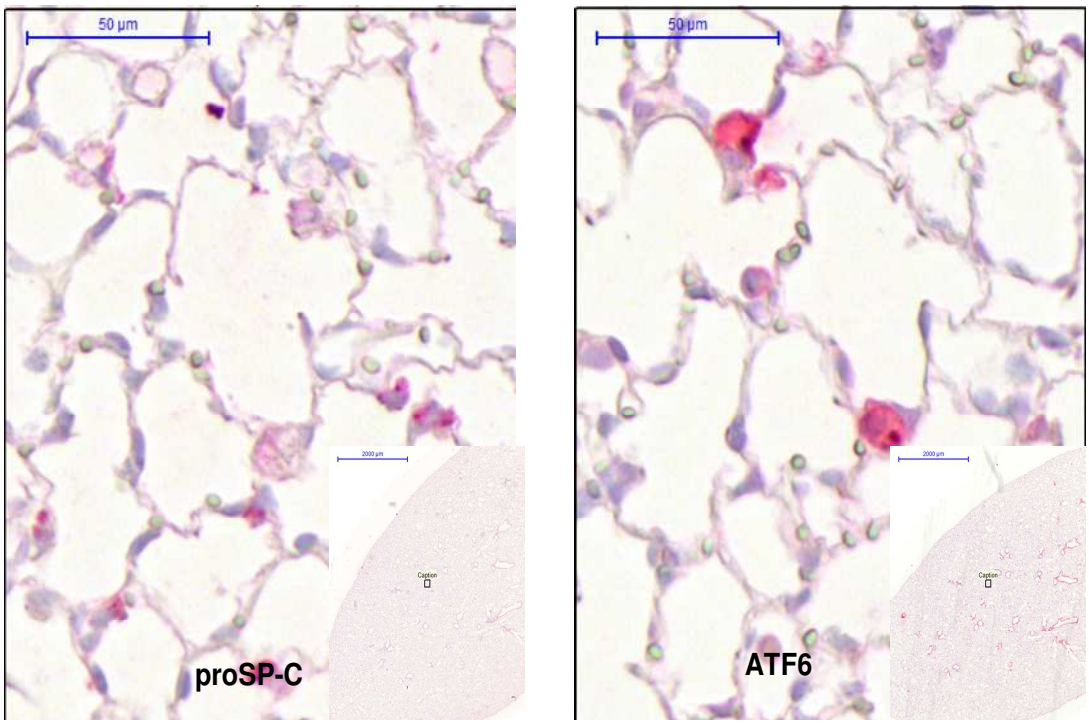
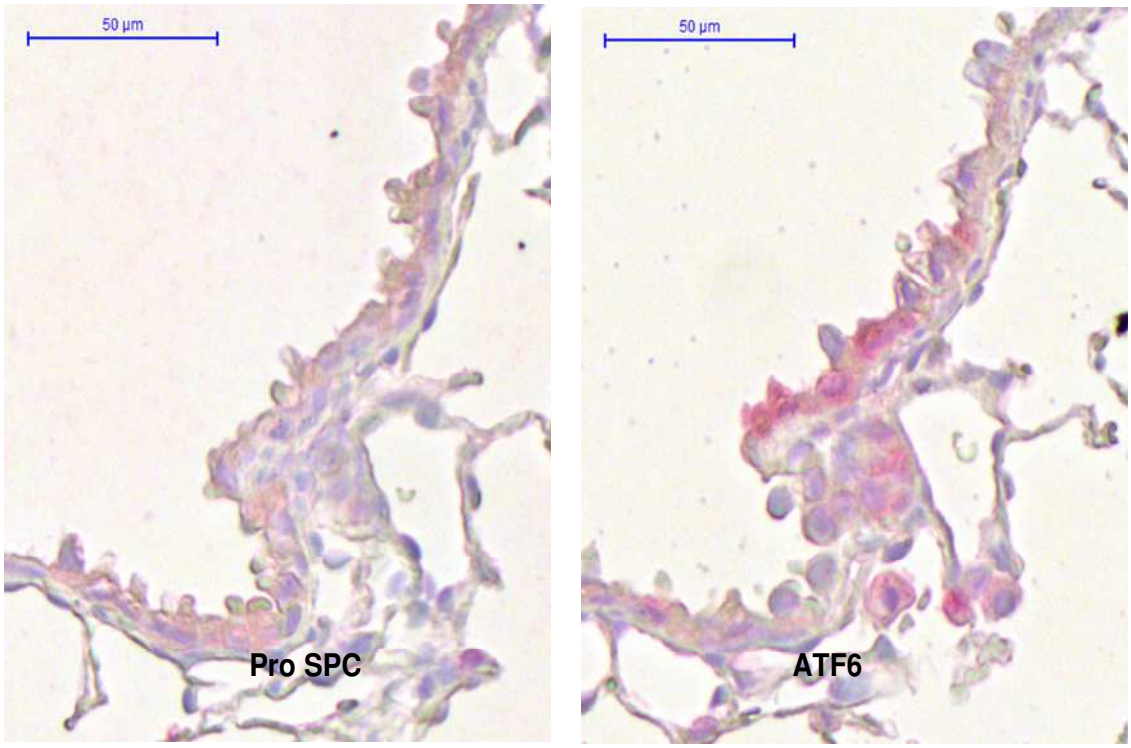


***Figure 11*: Immunohistochemistry of ATF6 (A-E) and Xbp1 (F-H)-mice (continued)**

Continue *figure 11*:

D) ATF6 transgenic mice,+-, 4 weeks on doxycyclin



E) ATF6 transgenic mice,+-, 4 weeks on doxycyclin

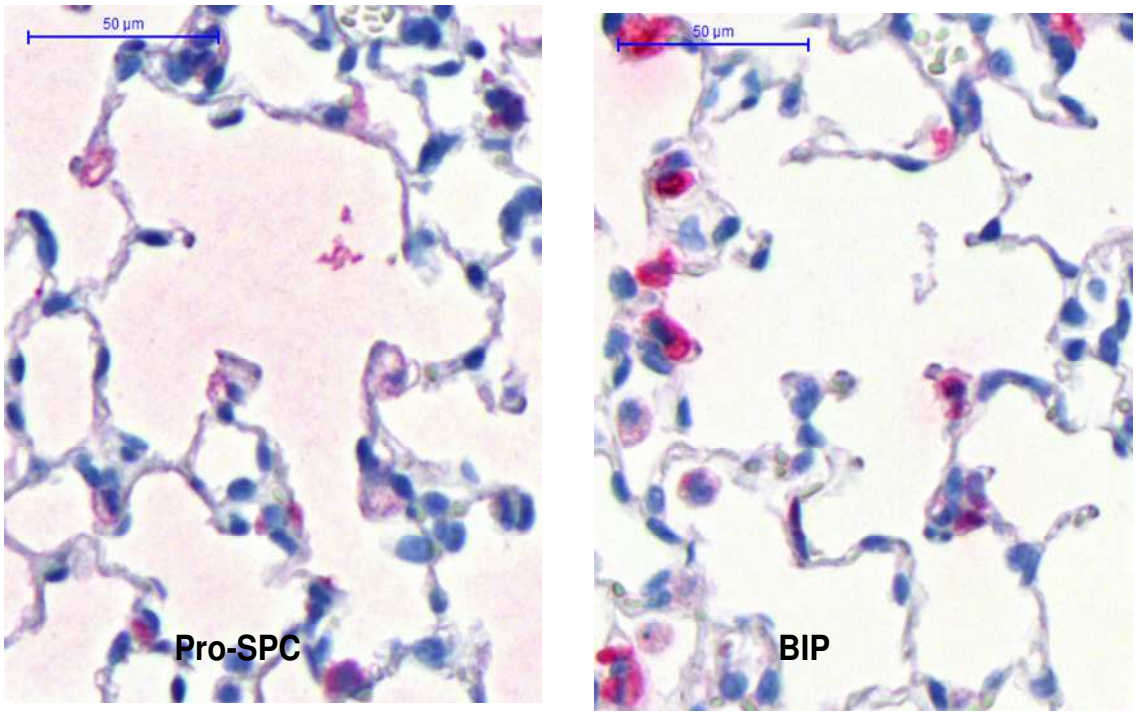
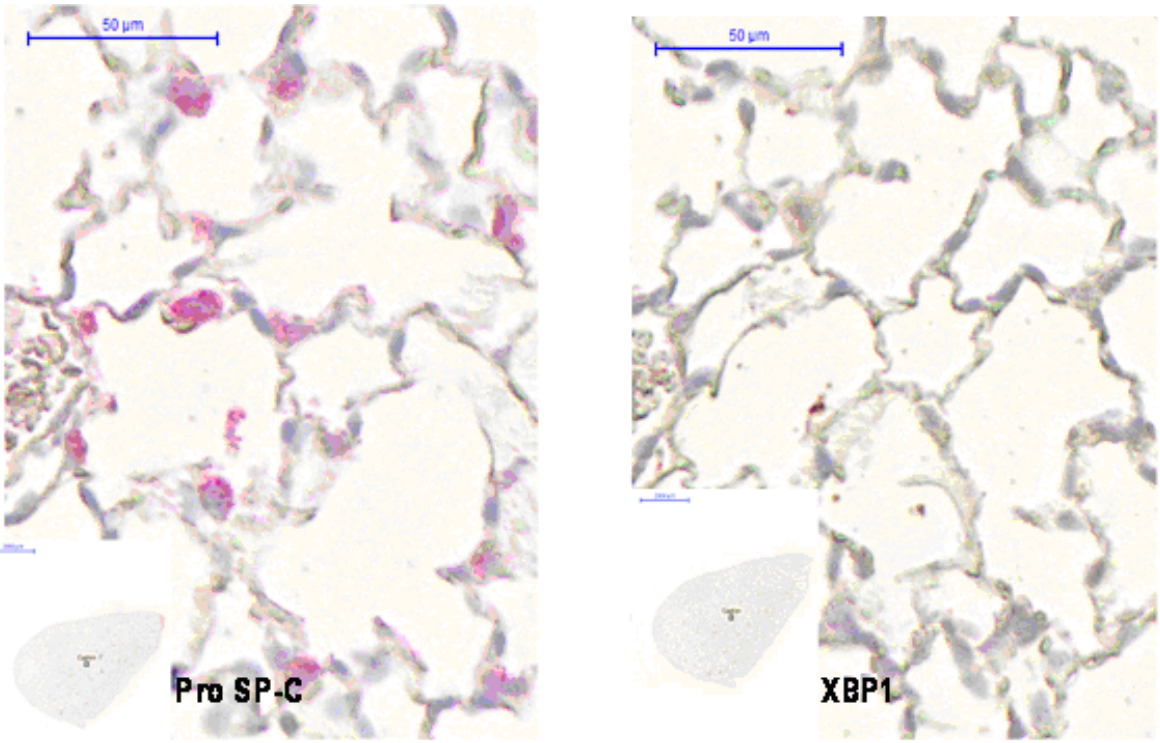


Figure 11

: Immunohistochemistry of ATF6 (A-E) and Xbp1 (F-H)-mice (continued)

Continue *figure 11*:

F) Xbp1 transgenic mice,+-, no induction with doxycyclin



G) Xbp1 transgenic mice,+-, 4 weeks on doxycyclin

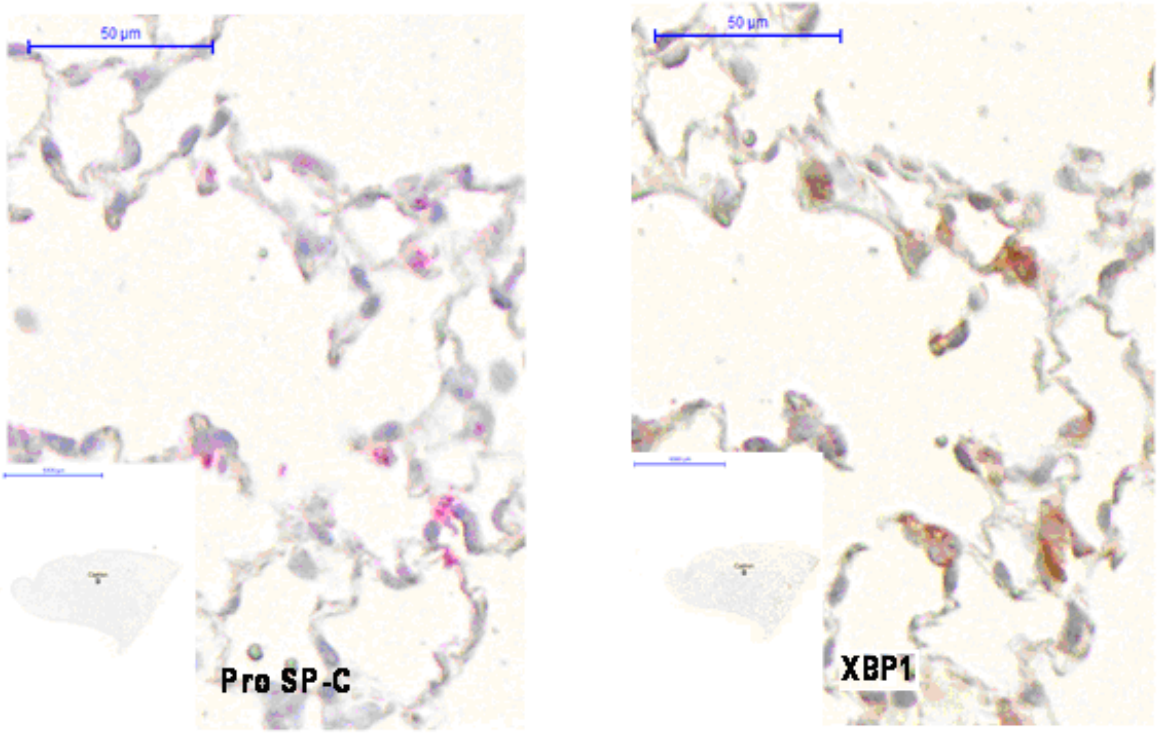


***Figure 11*: Immunohistochemistry of ATF6 (A-E) and Xbp1 (F-H)-mice (continued)**

Continue *figure 11*:

H) Xbp1 transgenic mice,+-, 4 weeks on doxycyclin

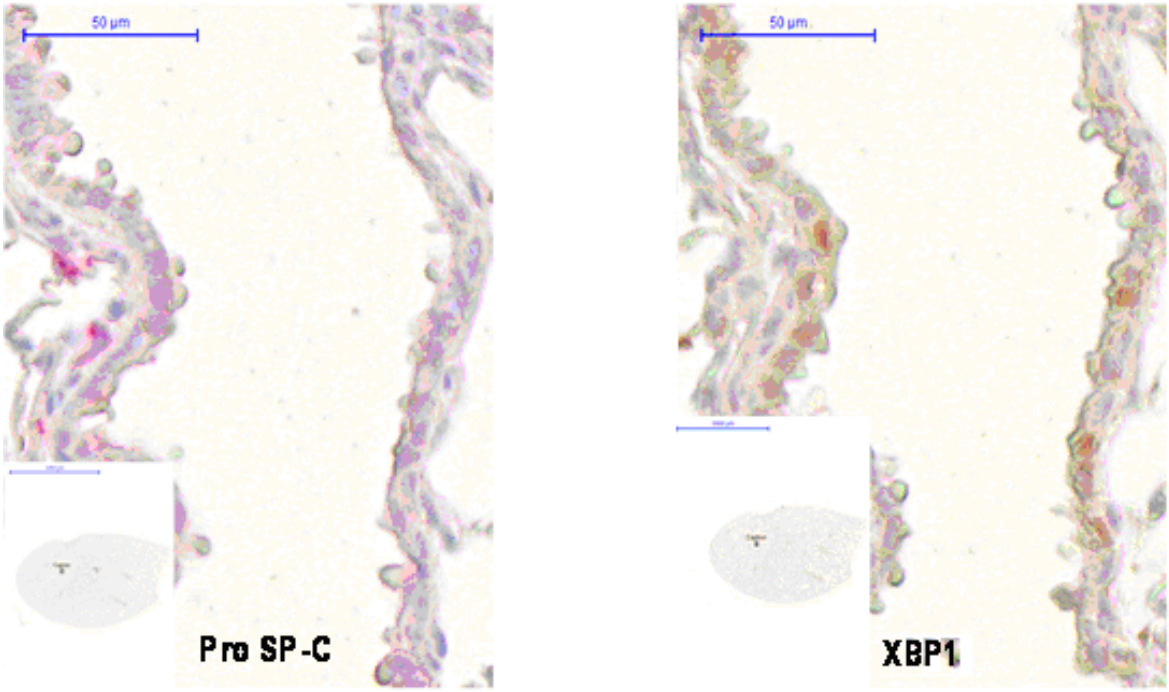


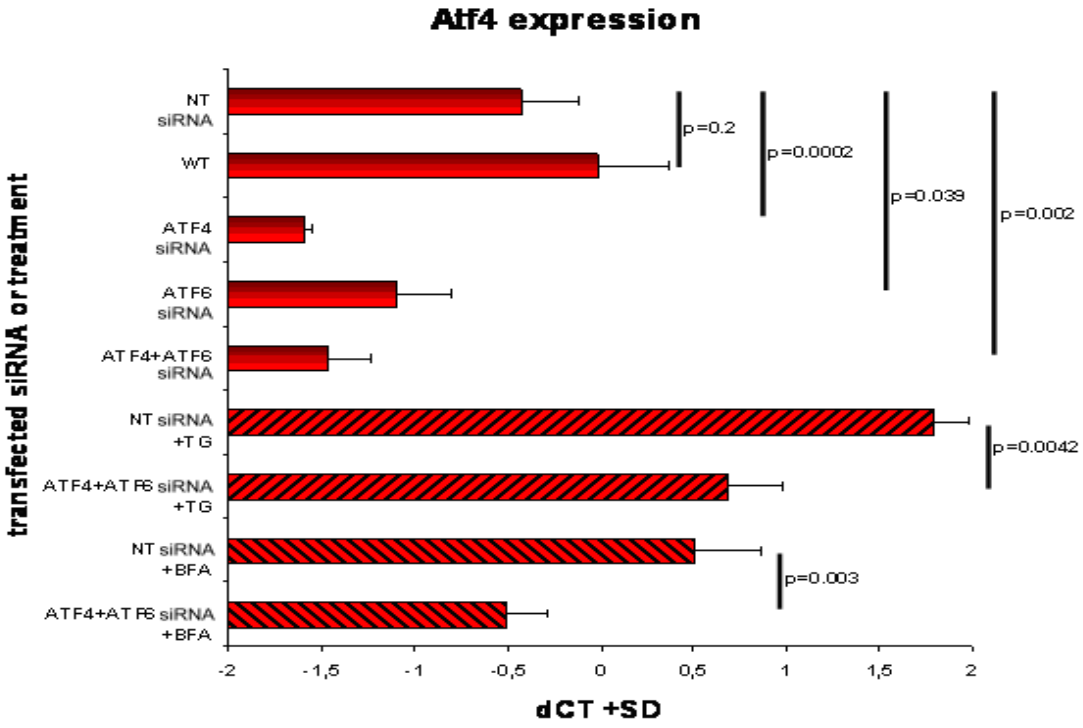
***Figure 11*: Immunohistochemistry of ATF6 (A-E) and Xbp1 (F-H)-mice.** Representative examples for Immunostainings of ATF6 and Xbp1 mouse lung slides. On the left hand are the (parallel) pro-SP-C stained sections on the right hand the sections stained for the antigen of interest (A-D: ATF6; E: BIP, F-H: XBP1). Staining for pro-SP-C, ATF6 and BIP was performed with fast red, staining for Xbp1 with DAP (brown). Nuclear staining was done with Haemalaun for 30 sec. A) FVBN-control, B) non-induced ATF6-mouse (4 weeks, +-), C) induced ATF6-mouse (4 weeks, +-), D) bronchiolar region of an induced ATF6-mouse (4 weeks, +-), E) induced ATF6-mouse (BIP-staining) (4 weeks, +-) (BIP staining of control mice developed for the same time was negative, data not shown). F) Non-induced Xbp1-mouse (4 weeks, +-), G) induced Xbp1-mouse (4 weeks, +-), H) bronchiolar region of an induced Xbp1-mouse (4 weeks, +-). Negative controls for BIP induction and the induction of ATF6 and XBP1 in the bronchiolar region are not shown. Enhanced ATF6 / Xbp1 expression is present in the Type II cells and a population of bronchial epithelial cells but not in the controls. Enhanced BIP expression was observed in the AECII of ATF6 over-expressing mice.

4.1.5 Functionality of siRNAs and effects of TG / BFA in siRNA-experiments

SiRNA-transfections of MLE12 were performed and assessed as described in the methods part (3.3.2). Atf4-siRNA transfection reduced the base line (NT, non-targeting siRNA) and the induced Atf4-mRNA-levels (BFA or TG [NT+BFA, NT+TG]) about one CT value and had a similar efficiency when used in

combination with Atf6-siRNA. Atf6-siRNA was as efficient as Atf4-siRNA. The effect on TG-induced Atf6-expression was slightly higher (about 1.5 CT-values difference). Singular siRNA transfection with Atf4-siRNA also significantly reduced Atf6 levels and Atf6-siRNAs also reduced Atf4 (off-target-effects). However, none of those siRNAs altered the level of (induced or non-induced) Xbp1(s). Treatment with TG strongly increased the Atf4-levels (about two CT-values) and the Xbp1(s)-levels (more than 5 CT-values) but hardly the level of Atf6 (about 0.5 CT-values). BFA only moderately increased Atf4 levels (about 0.5 CT-values) and did not induce Atf6. However, the BFA-treatment was sufficient to increase the Xbp1(s) expression-level about two CT-values.

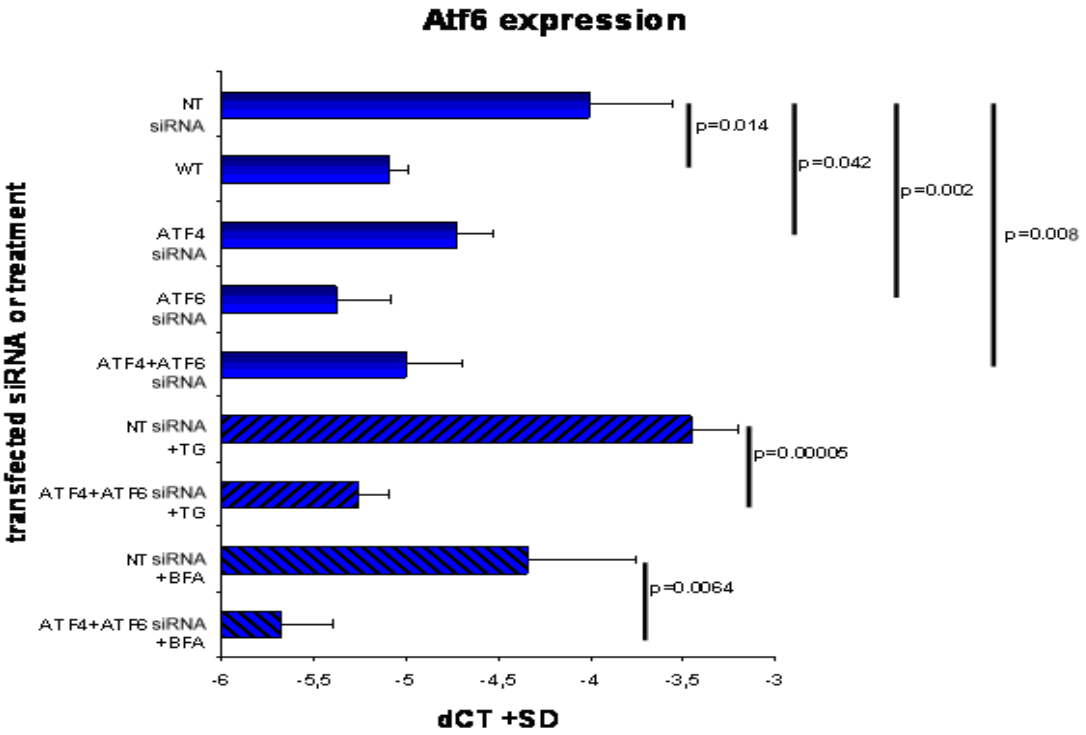
The changes in protein levels of ATF4 and ATF6 in this experiment were not assessed. However, cells treated with TG or BFA for 3 h in a control experiment did not reveal induction of p50ATF6 or induction of ATF4 by TG, but a robust protein induction of ATF4 by BFA (see *supplementary figure 2*).



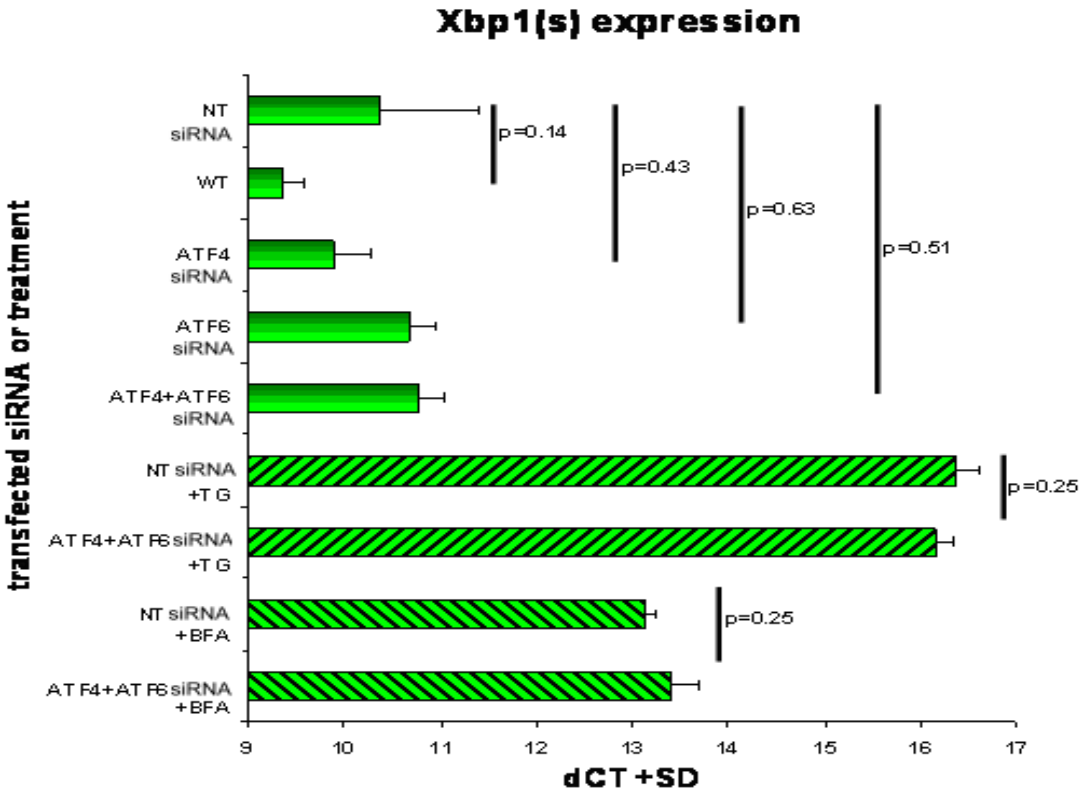
A

***Figure 12*: Silencing of Atf4 and Atf6 *in vitro* (continued)**

Continue *figure 12*:



B



C

***Figure 12*: Silencing of Atf4 and Atf6 *in vitro* (continued)**

***Figure 12*: Silencing of Atf4 and Atf6 *in vitro*.** MLE12 cells were transfected with siRNA against ATF4 or ATF6 or a combination of both. NT (non-targeting siRNA) served as a reference, WT (untreated) as an additional control (massive bars). Treatment with Thapsigargin (TG) or Brefeldin A (BFA) was held up for 2 h and started after a preincubation either with NT or Atf4 / Atf6 siRNA combitransfection (dashed bars). The harvested and reverse transcribed RNA was subject to qPCRs for Atf4 (A), Atf6 (B) or Xbp1(s) (C); n=4.

4.2 Transgenic expression of Atf4, Atf6 and Xbp1 did not result in development of lung fibrosis *in vivo*

As discussed above, the extent of transgene induction is not fully assessed for the mouse lines mentioned above and additional lines for Atf4 and Xbp1 have not yet been subjected to experiments. Therefore, the following data on the effects of the transgenes has to be regarded as a preliminary result.

4.2.1 General characterization of *in vivo* models

With two exceptions no significant changes in body weight or levels and composition of immune cells in the BAL of induced vs. non-induced transgenic mice could be detected. The weight of the three animal lines developed inconspicuous (see *supplementary table 2*). The cell numbers in the BAL were very different between individual animals and also between the experimental groups (Range: 57500-429900) but no systematic changes could be observed (*see supplementary table 3*, upper part). The BAL of mice which were 4 weeks in the experiment revealed an average macrophage content of 95.2 %-99.7 %. In the elder groups the average macrophage count ranged at 82.5 %-95 %, the rest representing lymphocytes and PMN (polymorph nuclear cells). However, most of the individual elder mice also had macrophage counts of 98-100 % (see *supplementary table 3*, lower part).
During the experimental phase of feeding with Dox, four out of 115 mice and out of four different groups died. Two of them were induced, two of them Atf4-transgenic mice and another two Atf6-transgenic-mice (see *supplementary table 4*). The average lung compliance ranged between 5.7 and 9.7 ml/kPa*Kg and was typically increased with age (see *supplementary table 5*). The average running time on the treadmill increased between 0.7 and 7.6 min during the experiment and maximum exercise was observed in some cases after four, in others after sixteen or 32 weeks (see *supplementary table 6*). Within the different lines, there were no

significant differences with regard to death, lung compliance and running time as a result of Dox feeding (*supplementary tables 4, 5 and 6*).
The appearance of the lungs in histologic slides was rather normal and differences between induced and uninduced animals (of any line) were not observed (see *figure 13*). An exception of the general normal appearance of the lungs was the “canon shot” phenotype, discussed in the next chapter (4.3.2). IHC-detection of CHOP and cleaved caspase-3 did not reveal a specific signal in induced and non-induced Atf6 and Xbp1-mice but was detected in some cells of BLEO-treated mice, stained in parallel (Data not shown).

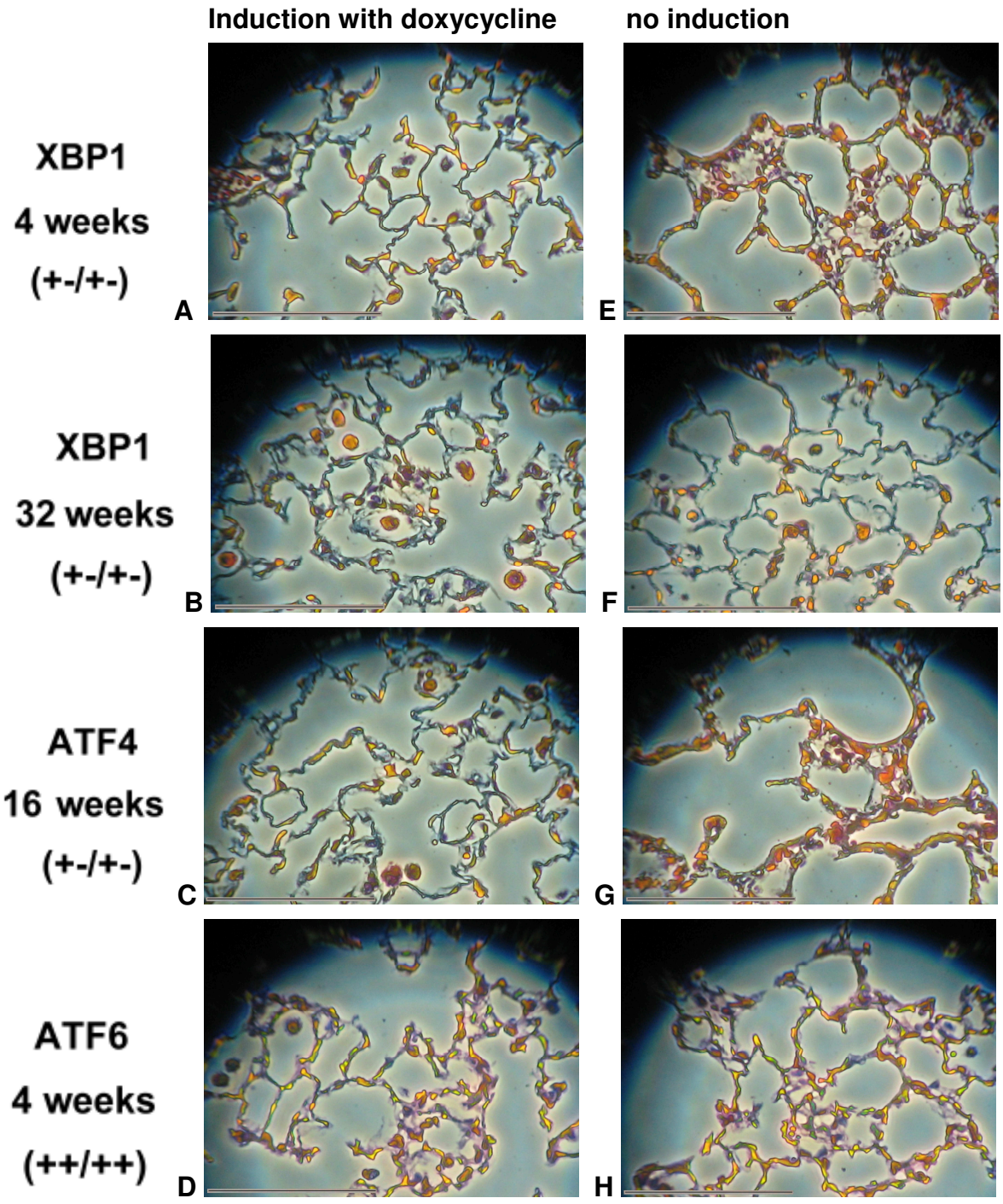


***Figure 13*: Morphology of transgenic mouse lungs.** The images are representative examples for the morphology of transgenic animal lungs with (A-D) and without (E-H) Dox-induction. A+E: +-/+- Xbp1 mice after four weeks; B+F: +-/+- Xbp1 mice after 32 weeks; C+G: +-/+- Atf4 mice after sixteen weeks; D+H: ++/++ Atf6 mice after 4 weeks. Scalebar: 100 µm, staining: Trichrome.

4.2.2 The “canon shot“-phenotype

In some microscopic lung sections from experimental animals, a special phenotype was observed, which is here addressed to as “canon shot” phenotype. The lungs had holes, typically appearing perfectly round.

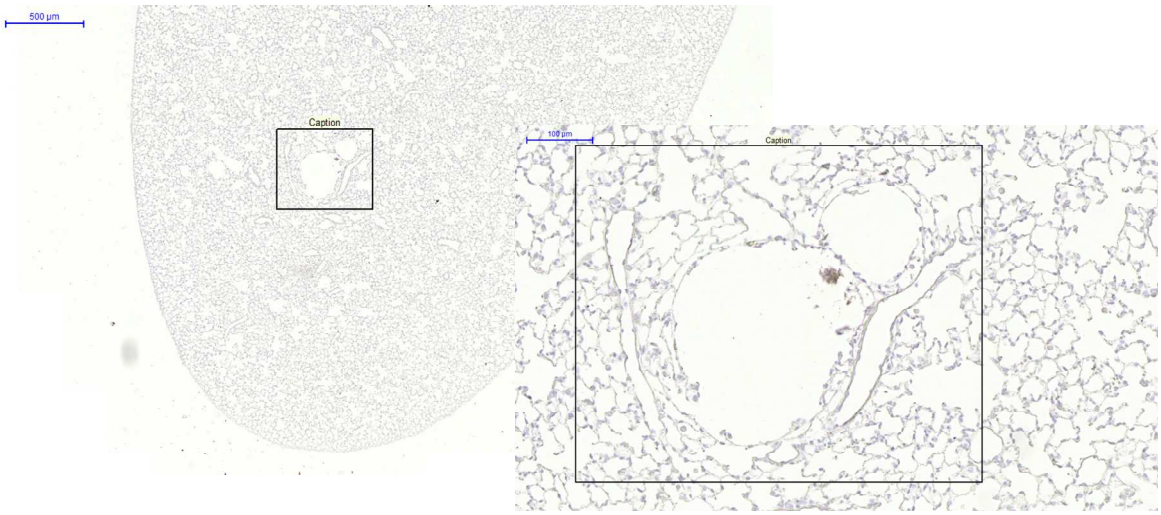


***Figure 14*: The “canon-shot” phenotype.** Example for a “canon-shot” in an Atf4 and SP-C-rtTA- +-, non-induced mouse. Age: 12-weeks. Scalebars: 500 µm (overview) and 100 µm (caption). Staining: Haemalaun.

To elucidate what might be the cause of the phenotype, the available IHC, Trichrome and H&E stained slides were searched for the phenotype. The findings are listed in *table 2* below.

***Table 2*: Frequency of the "canon-shot" phenotype**

	Canonshot observed	Animals in the group	%
Total	29	85	34.12
ATF4	11	40	27.50
ATF6	11	20	55.00
XBP1	7	25	28.00
induced	15	43	34.88
non induced	14	42	33.33
in ++/++ animals	5	10	50.00
in +-/+- animals	24	75	32.00
in 4 weeks group	14	40	35.00
in older mice	15	45	33.33

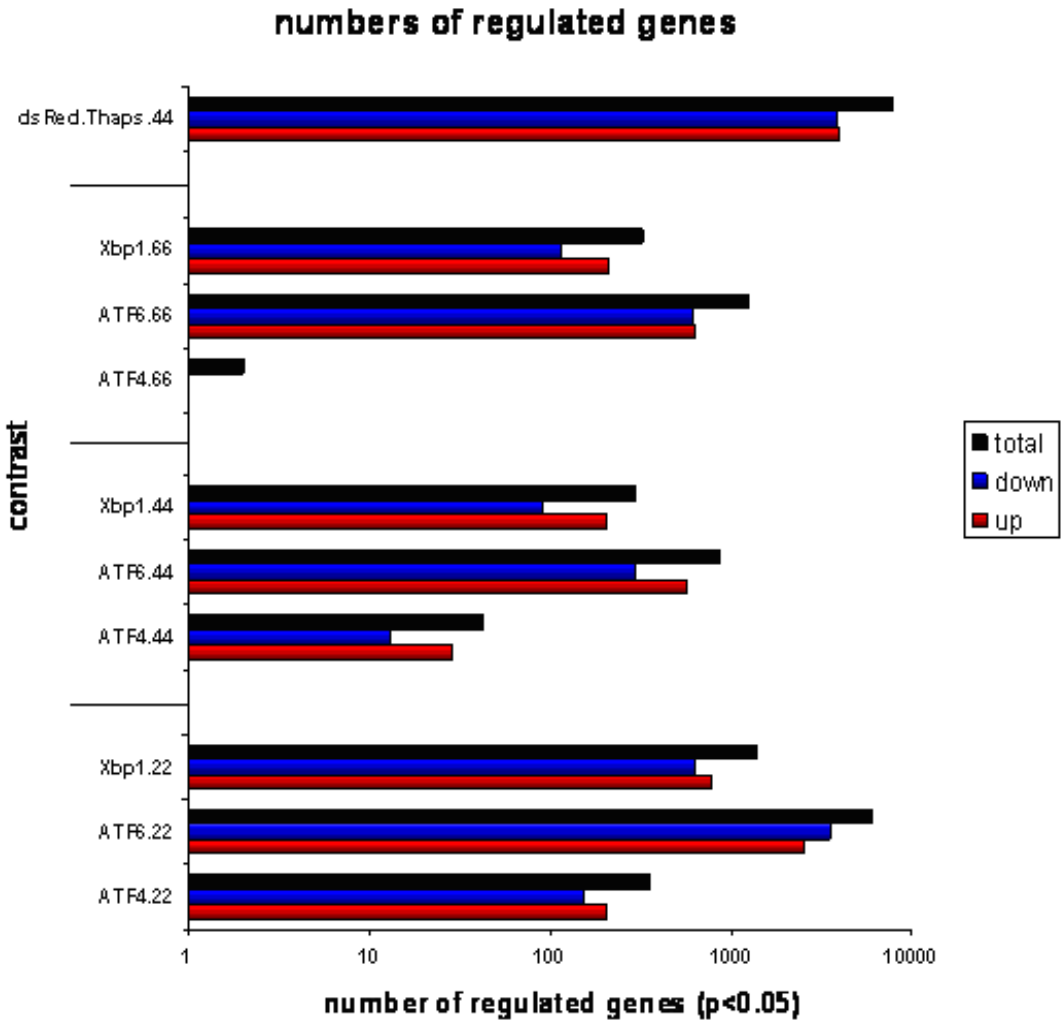
The phenotype was not observed in lungs from nine different founders and several SP-C rtTA negative offspring and (with one exception) not in FVBN mice.

The common feature of the mice with the phenotype is the existence of the transactivator (SP-C rtTA), not Dox or the induction or presence of one of the transgene-inserts.

4.3 ER-stress and UPR *in vitro*: a general characterization based on microarrays

4.3.1 Effects of Atf4, Atf6 and Xbp1 on mRNA expression in MLE12 cells

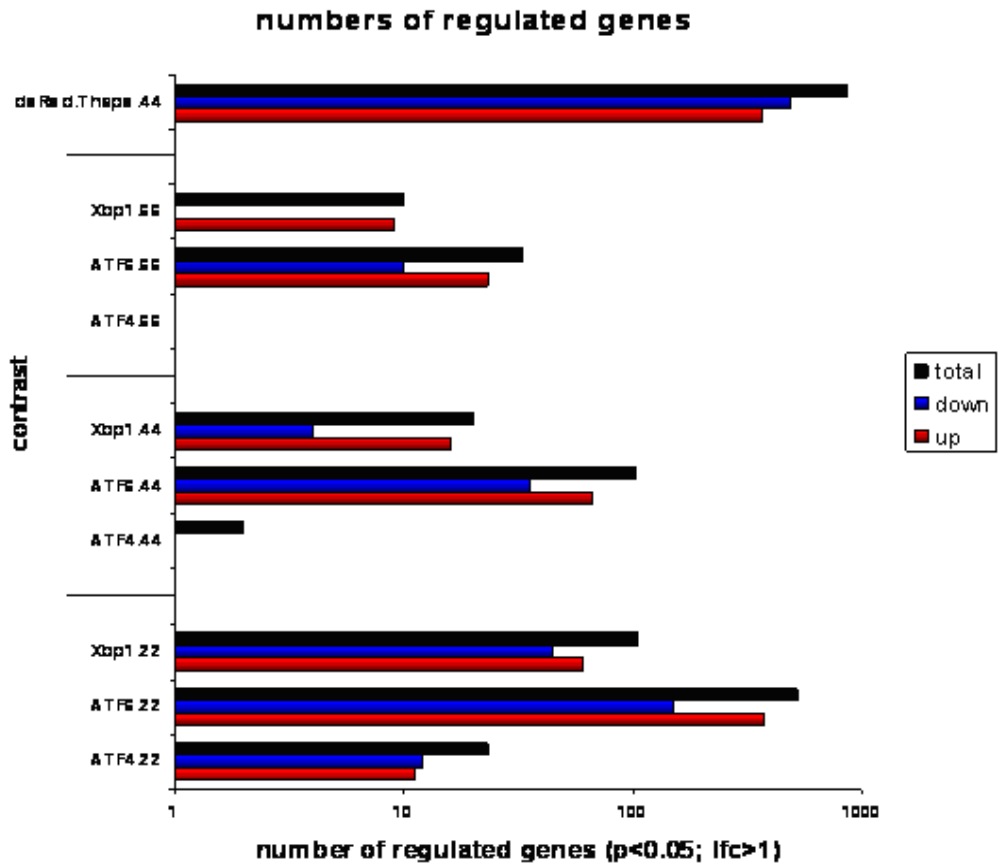
13254 probes were regulated significantly by Atf4, Atf6, Xbp1 and / or TG. 1362 of those genes were at least regulated with a log fold change (lfc) of $\pm$1. A complete list of regulated genes exceeds the space of this work but an excerpt from the microarray results, containing such genes which gain importance in the discussion can be found in the appendix (S*upplementary table 7*). TG was leading to the strongest regulation, followed by Atf6 and Xbp1. The initially high(er) number of regulated downstream genes of Atf4, Atf6 and Xbp1 was declining 44 h and 66 h after transfection (see *figure 15*).



A

***Figure 15*: Extend of the response of MLE12 cells to UPR- transcription factor over-expression and Thapsigargin treatment (continued).**

Continue *figure 15*:



B

***Figure 15*: Extend of the response of MLE12 cells to UPR- transcription factor over-expression and Thapsigargin treatment.** A) Numbers of significantly regulated genes. B) Numbers of strongly (lfc>1) and significantly regulated genes. The logarithmic scale depicts zero as one.

A heat map of the strongly regulated genes reveals a similar signalling of Atf4, Atf6 and Xbp1 if we look at the same time point after transfection. The clustering and inclusion of the depicted genes of the two heat maps depends on the included contrasts, leading to a diverse appearance (*figure 16*). But the early signalling of the transgenes (22 h) is obviously partly the reverse than the later signalling (44 h and 66 h), indicating a counter regulation against the initial effects. We can also observe similarities and dissimilarities between TG treatment of diverse duration and transgene effects.

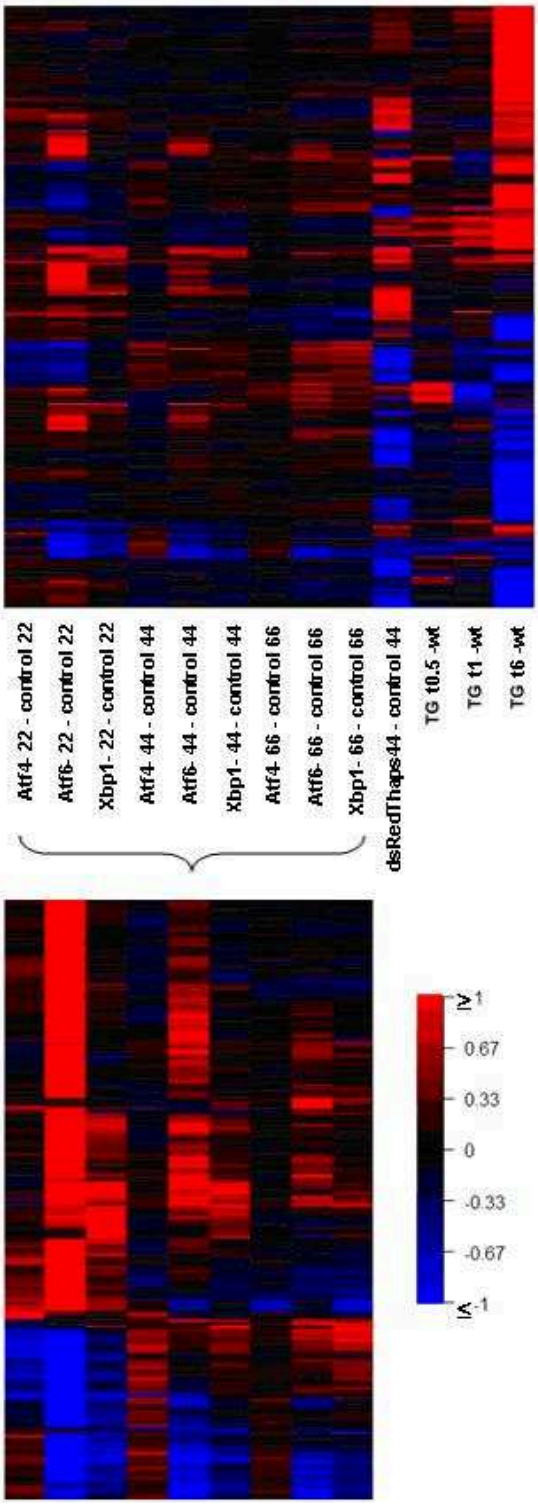
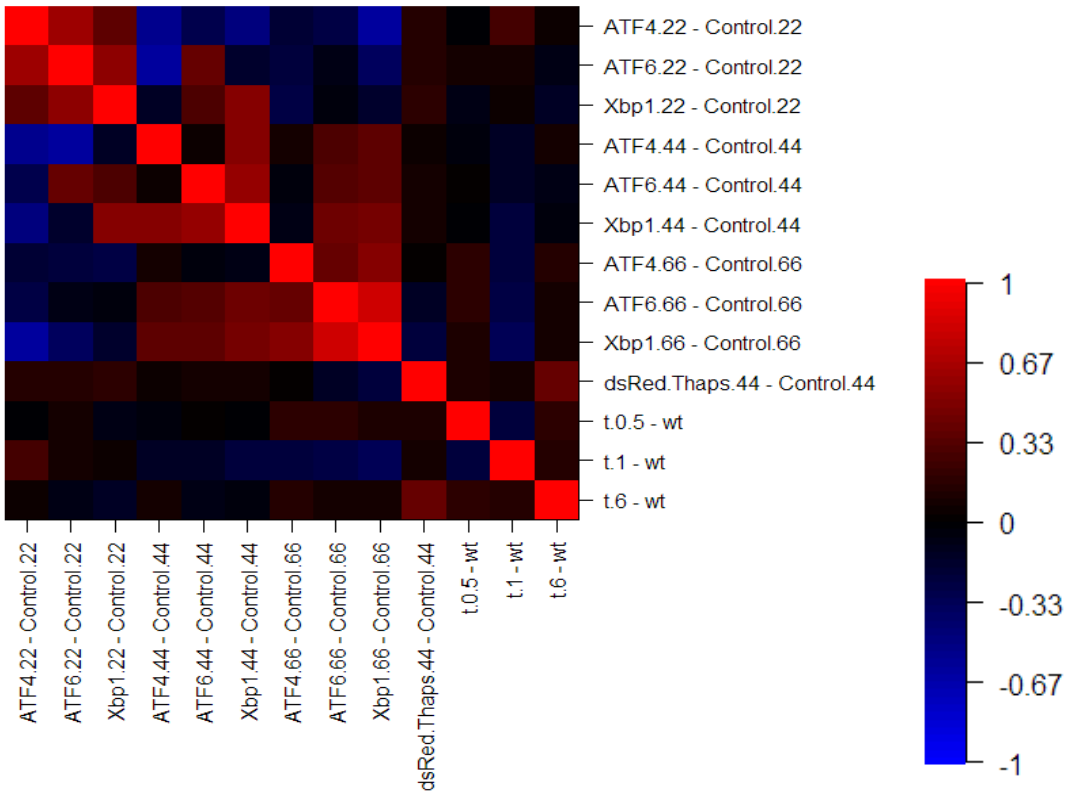


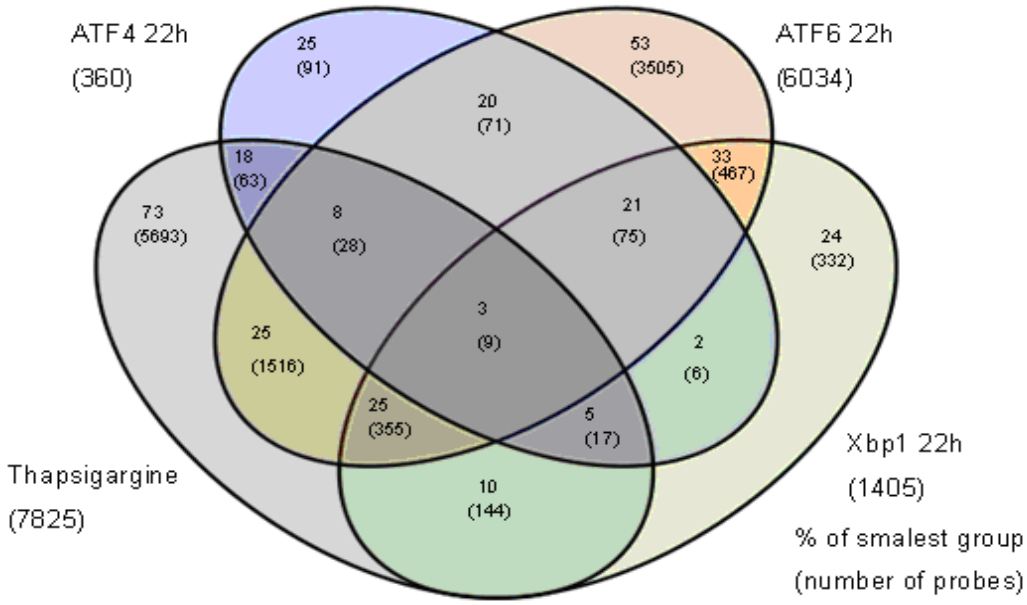
***Figure 16*: Time dependent UPR-transcription factor and Thapsigargin effects.** The upper heat map includes the effects of TG and the effects of the transgenes (selection for both: genes with $p \leq 0.05$ and $lfc \geq 1$ in one contrast). In the lower one, exclusively the effects of the transgenes are depicted. The clustering and inclusion of the depicted genes of the two heat maps depends on the included (**continued)**

Continue *figure 16*: contrasts, leading to a diverse appearance. DsRedThaps44 – control 44 stands for 22 h TG treatment after 22 h of transfection with the pIRES2dsRed2-vector. TG t0.5, TG t1 and TG t6 stands for 0.5, 1 and 6 h of TG treatment of non-transfected cells. “Control” stands for pIRES2dsRed2-transfected cells and WT for untreated cells.

The correlation chart depicted in *figure 17 A* shows a moderate positive correlation between the early transgene effects and the effect of TG which is also reflected in the Venn-diagram below. This correlation in between the transgene effects is much stronger for the down-regulated genes (*figure 17 C*). On the other hand, the stronger correlation between transgenes and TG can be observed for the up-regulated genes (*figure 17 B*). Focusing on the very early TG-effects (t0.5) and the early effects of Atf4, Atf6 and Xbp1, we cannot observe a robust correlation in between these contrasts. However, if we limit our observation on the up-regulated genes, the correlation of the early TG effects especially with the Atf4-effects is much more pronounced.

Significiantly regulated



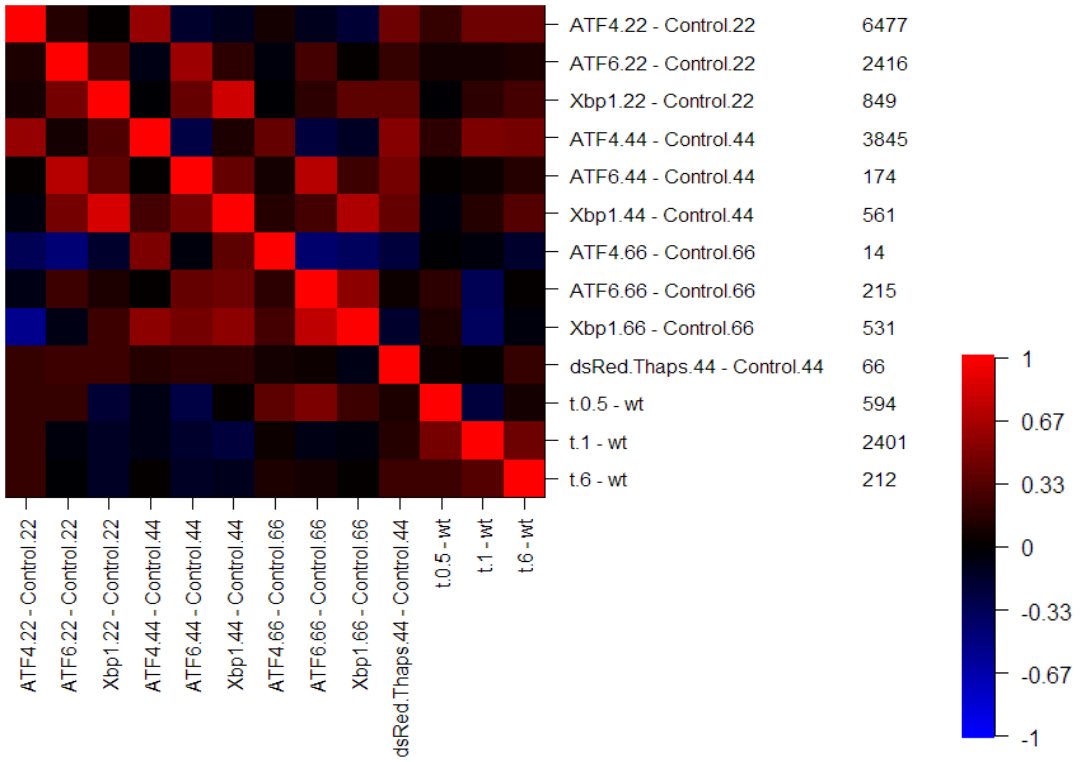


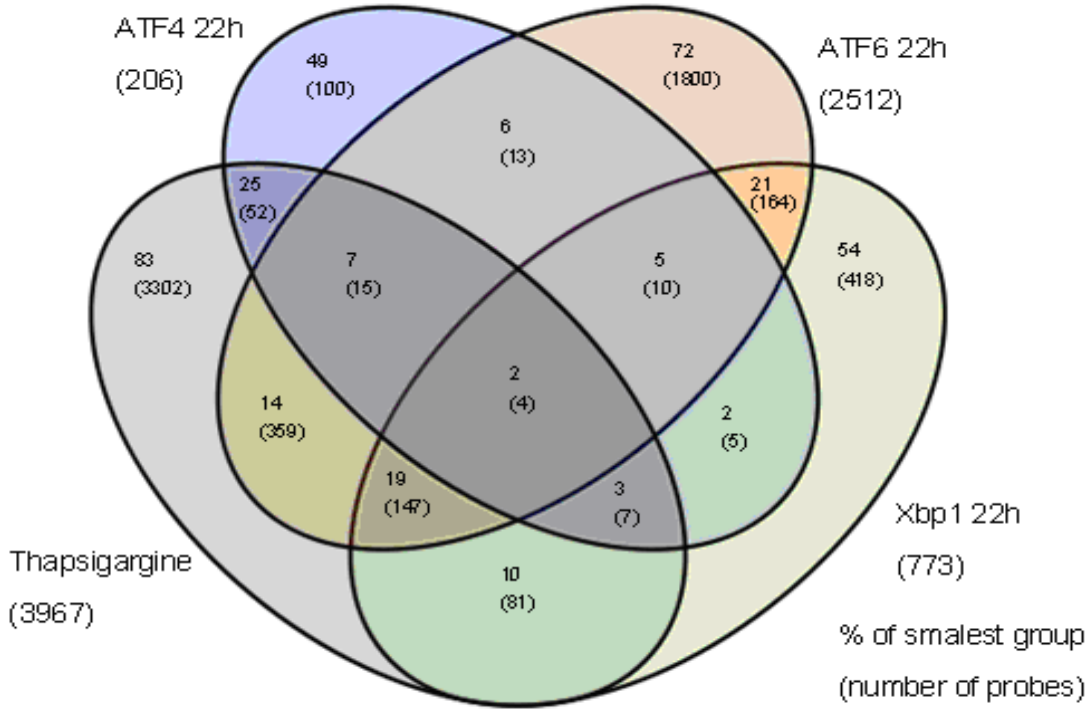
A

***Figure 17*: Correlation of the effects elicited by over-expression of ATF4, ATF6 and XBP1 and TG-effects (continued).**

Continue *Figure 17*:

Significantly up-regulated



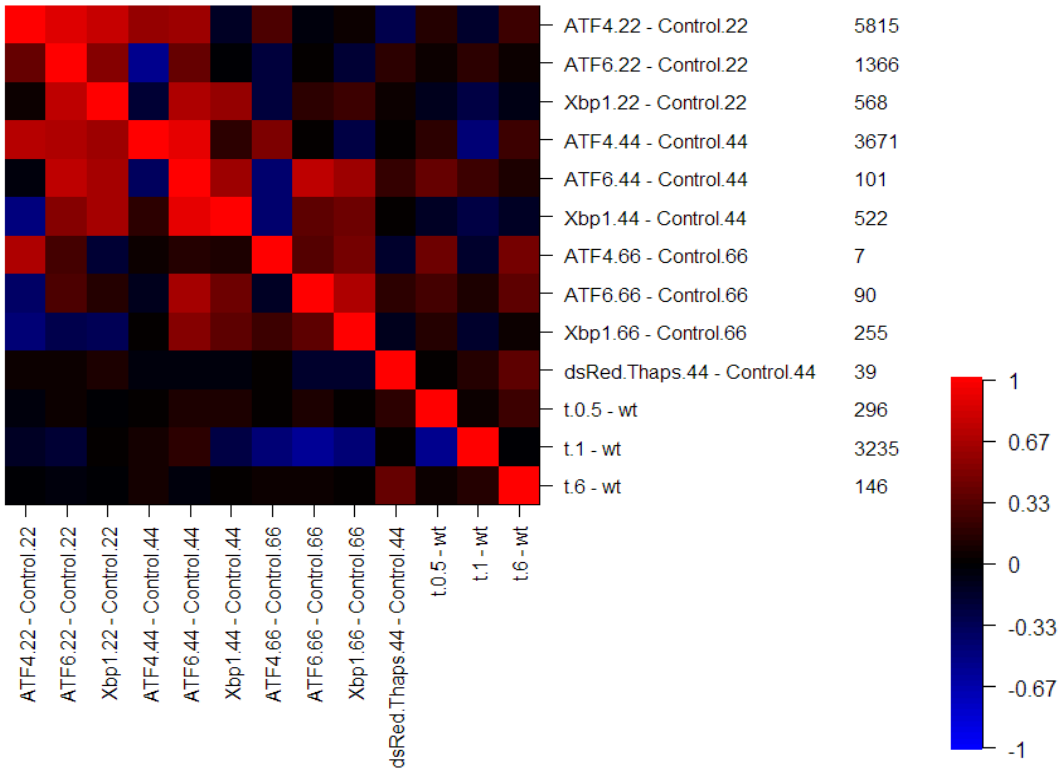


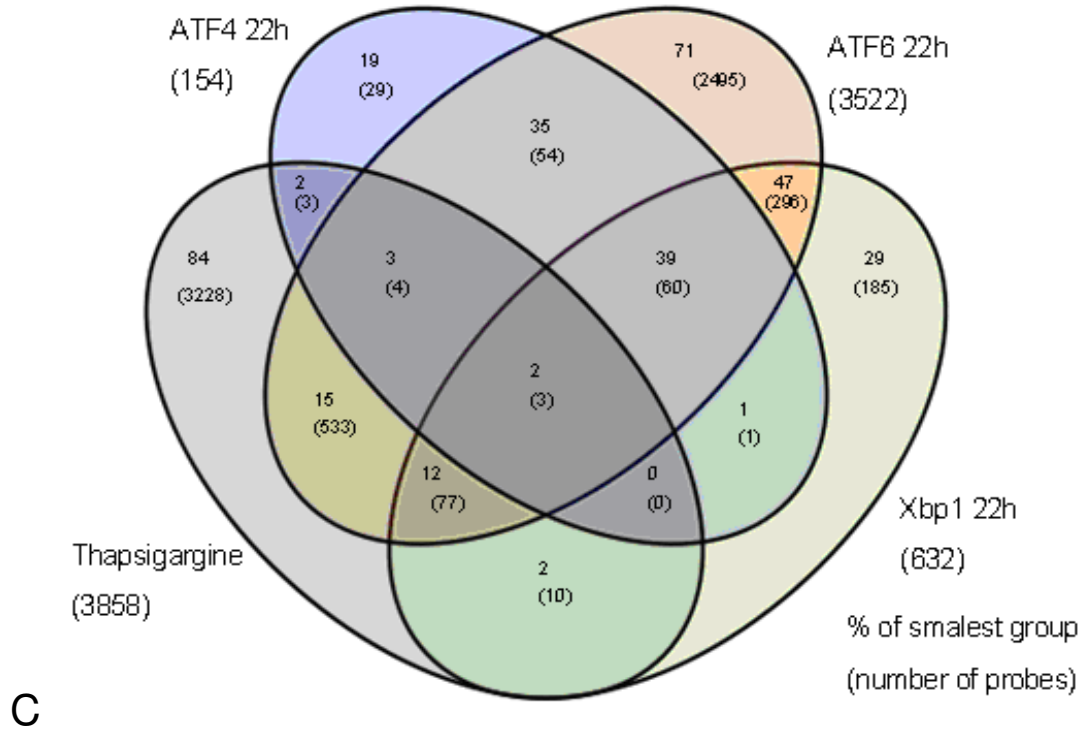
B

***Figure 17*: Correlation of the effects elicited by over-expression of ATF4, ATF6 and XBP1 and TG-effects (continued).**

Continue *Figure 17*:

Significantly down-regulated





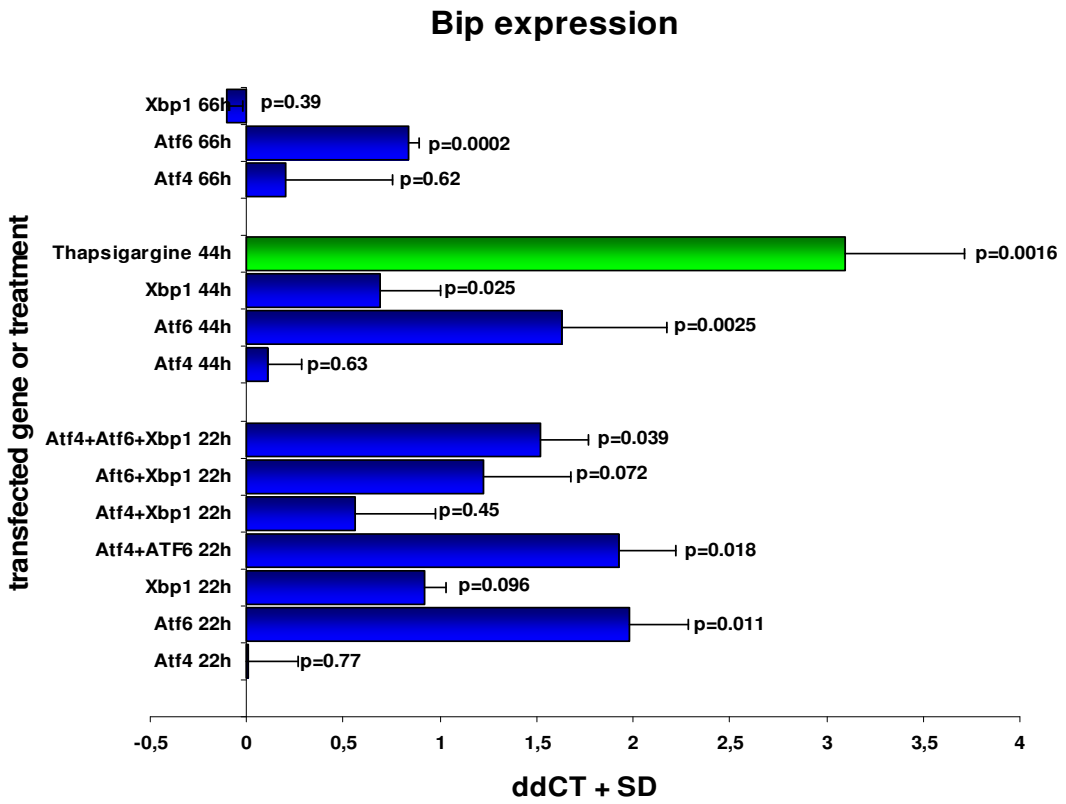
C

***Figure 17*: Correlation of the effects elicited by over-expression of ATF4, ATF6 and XBP1 and TG-effects (continued).**

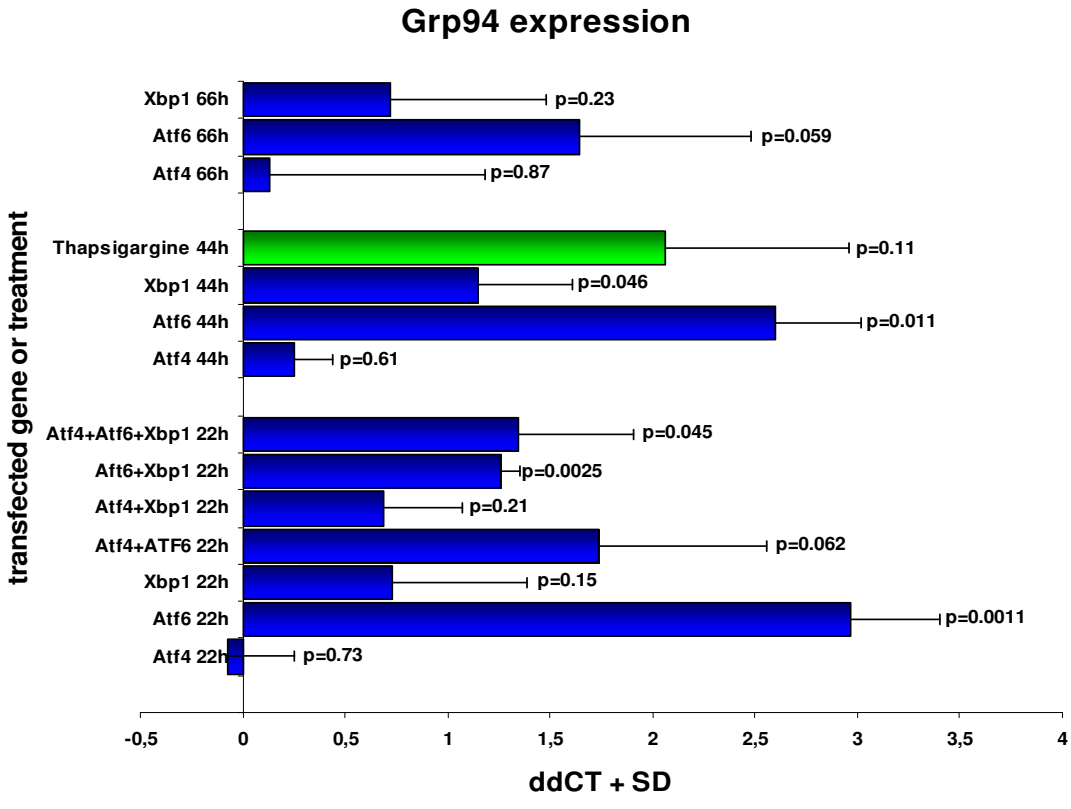
***Figure 17*: Correlation of the effects elicited by over-expression of ATF4, ATF6 and XBP1 and TG-effects.** A) All significantly regulated genes are included ($p < 0.05$ and $lfc > 0$). B) Only up-regulated genes are included. On the right hand the numbers of included genes are depicted. C) Only down-regulated genes are included. On the right hand the numbers of included genes are depicted. The upper part of A, B and C are colour coded correlation charts representing all treatments and transfections: DsRedThaps44-control 44 stands for 22 h TG treatment after 22 h of transfection with the pIRES2dsRed2-vector. t0.5, t1 and t6 stands for 0.5, 1 and 6 h of TG treatment of non-transfected cells. "Control" stands for pIRES2dsRed2-transfected cells and WT for untreated cells. The lower parts of A, B and C are Venn-diagrams for the time point 22 h after transfection and DsRedThaps44 – control 44 depicting the numbers of the probes significantly regulated (in the indicated direction) and the percentage of the probes belonging to this group. Where percentages for overlaps are depicted they represented the smallest group. E.g. 20 % overlap for Atf4 and Atf6 depicted in A means that 20 % of the genes regulated by Atf4 are also regulated by Atf6, while a much lower percentage of the (larger number of) genes regulated by Atf6 are also regulated by Atf4.

The gene set enrichment analysis (GSEA) revealed that the gene regulation affected a lot of biochemical pathways. In total, 59 % of all investigated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were significantly regulated in at least one of the contrasts (adj. $p<0.05$, including the analysis of the early TG response) indicating a very broad spectrum of cellular responses to ER-stress (see *supplementary table 1*).

Some potential downstream genes of Atf4, Atf6 and Xbp1 were also investigated by qPCR. The regulation of the transgenes themselves and of Chop is shown elsewhere (4.4.1), results for the mRNA-regulation of Bip, Grp94, VCP, Ppib, Edem1, Atf3, Nfe2l2, and Bad, are summarized in *figure 18* (following pages). No significant regulations were found for Dnajc10, Atg12, Chkb and Nf-y a (Data not shown).



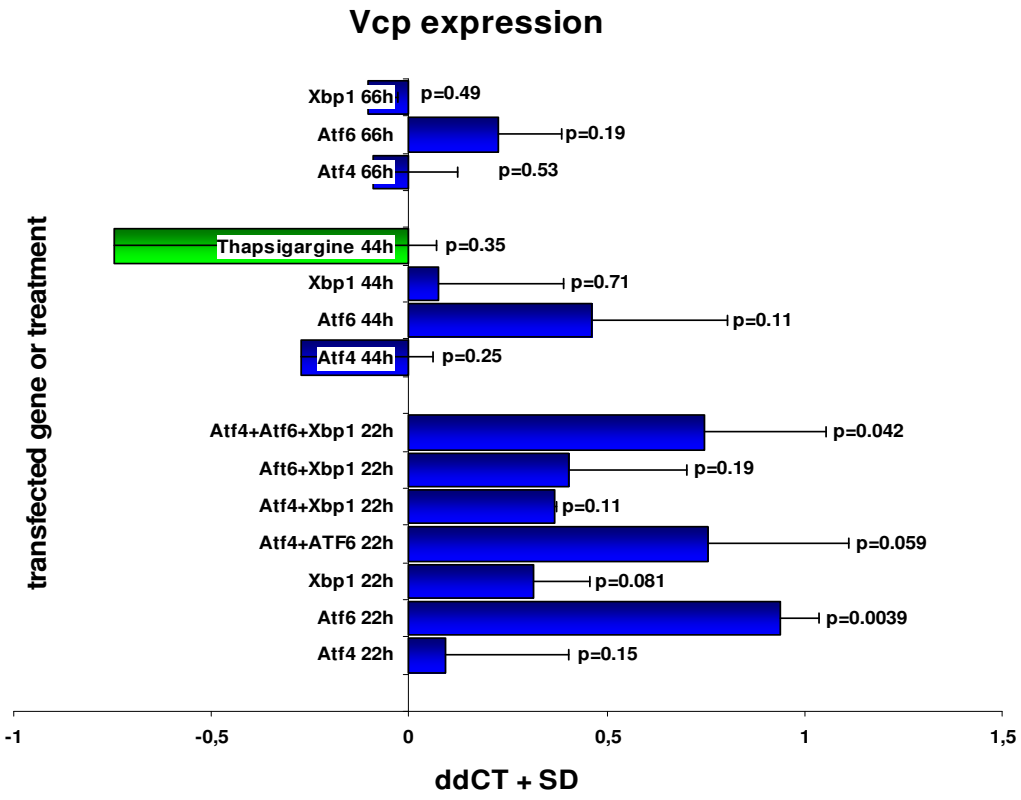
A



B

***Figure 18*: Verification of microarray results by qPCR (continued).**

Continue *Figure 18*:



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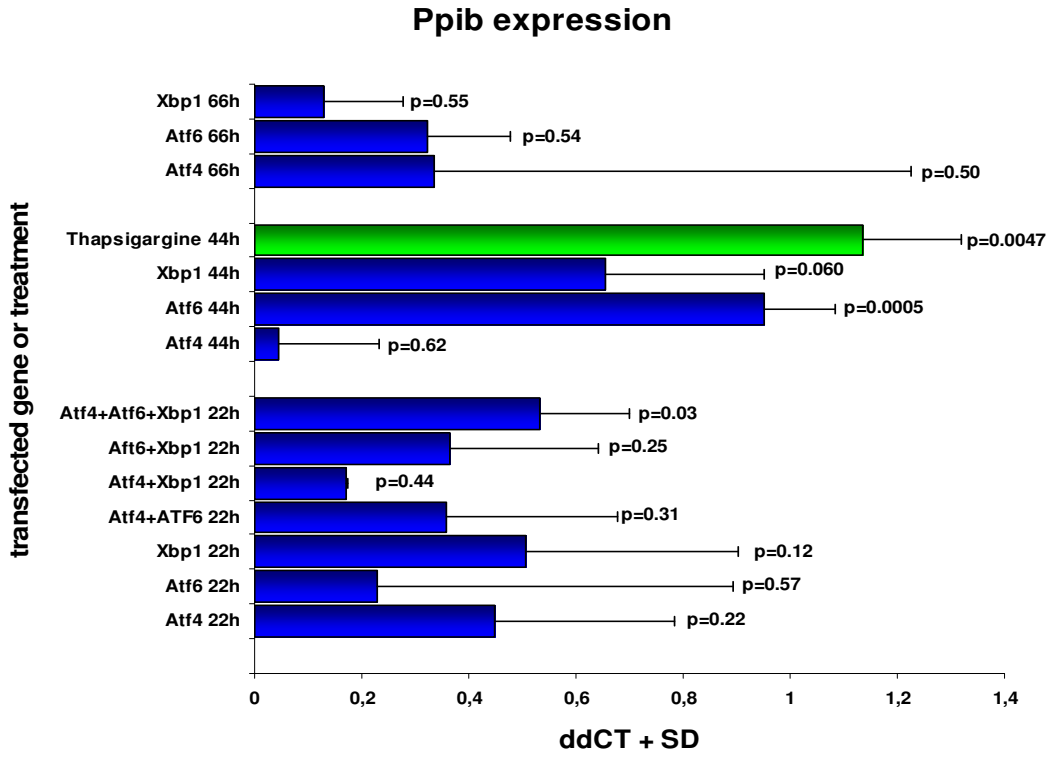
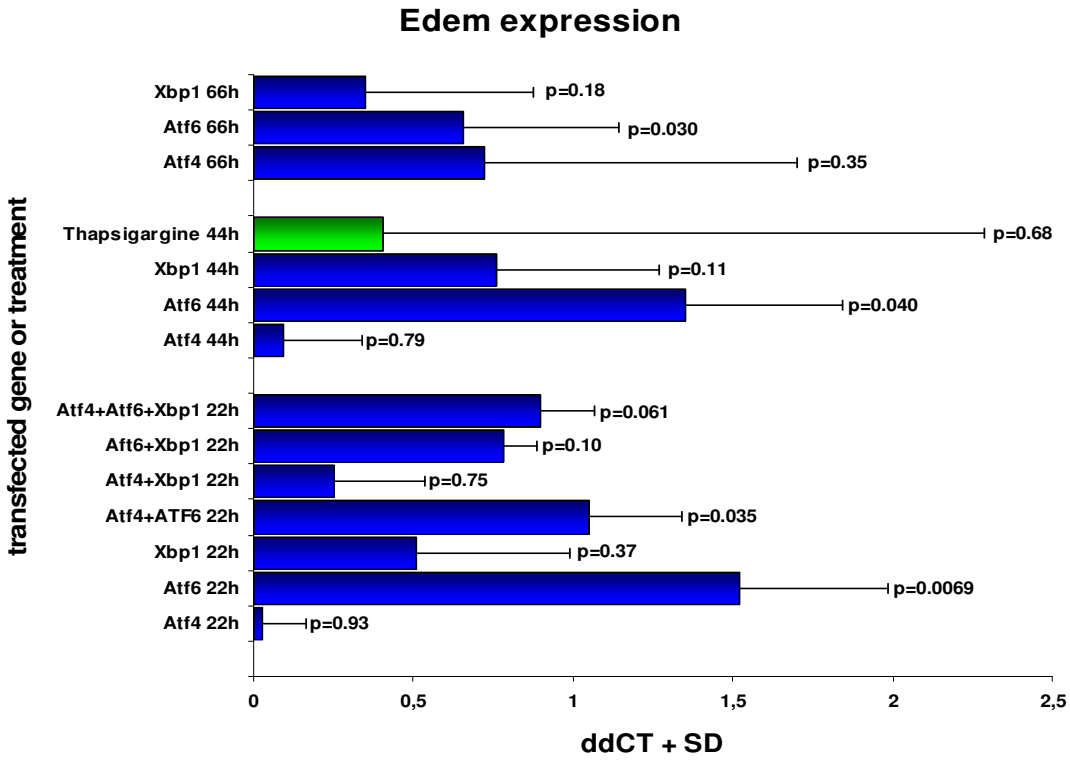


D

***Figure 18*: Verification of microarray results by qPCR (continued).**

Continue *Figure 18*:



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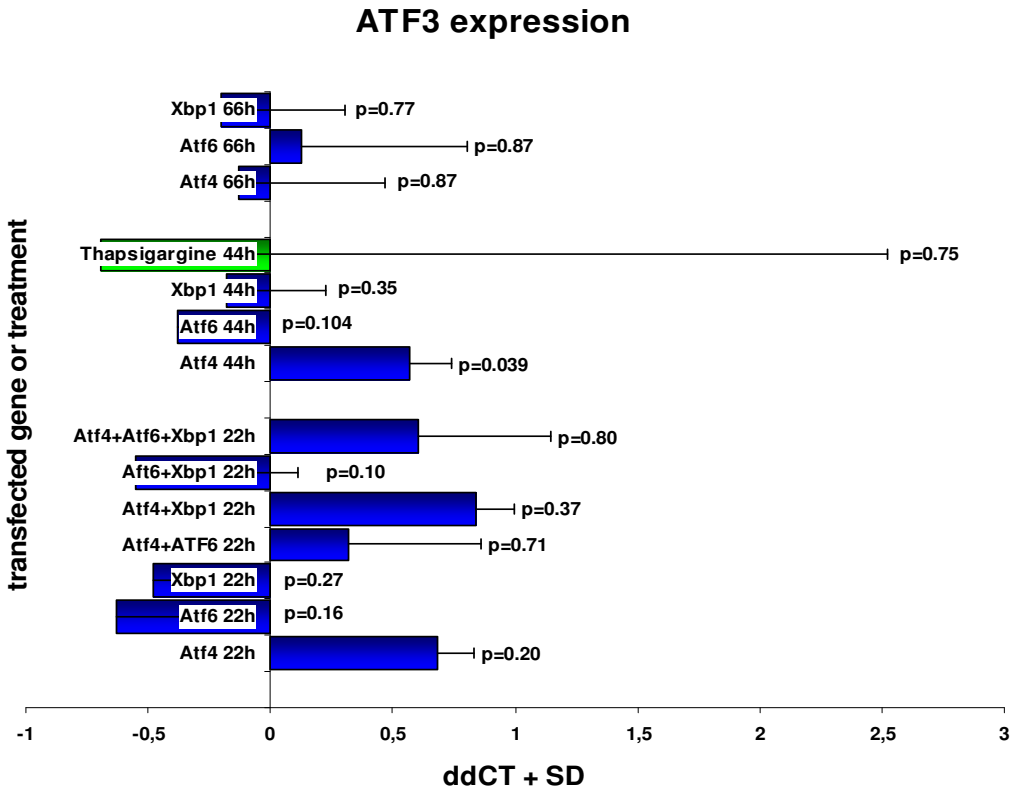
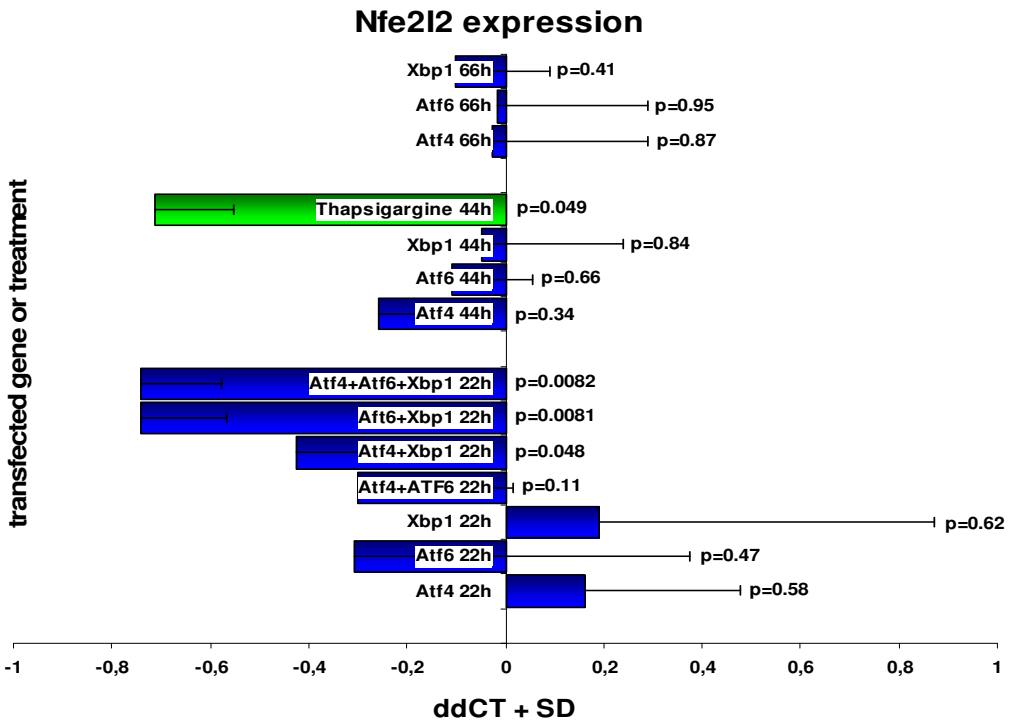


F

***Figure 18*: Verification of microarray results by qPCR (continued).**

Continue *Figure 18*:



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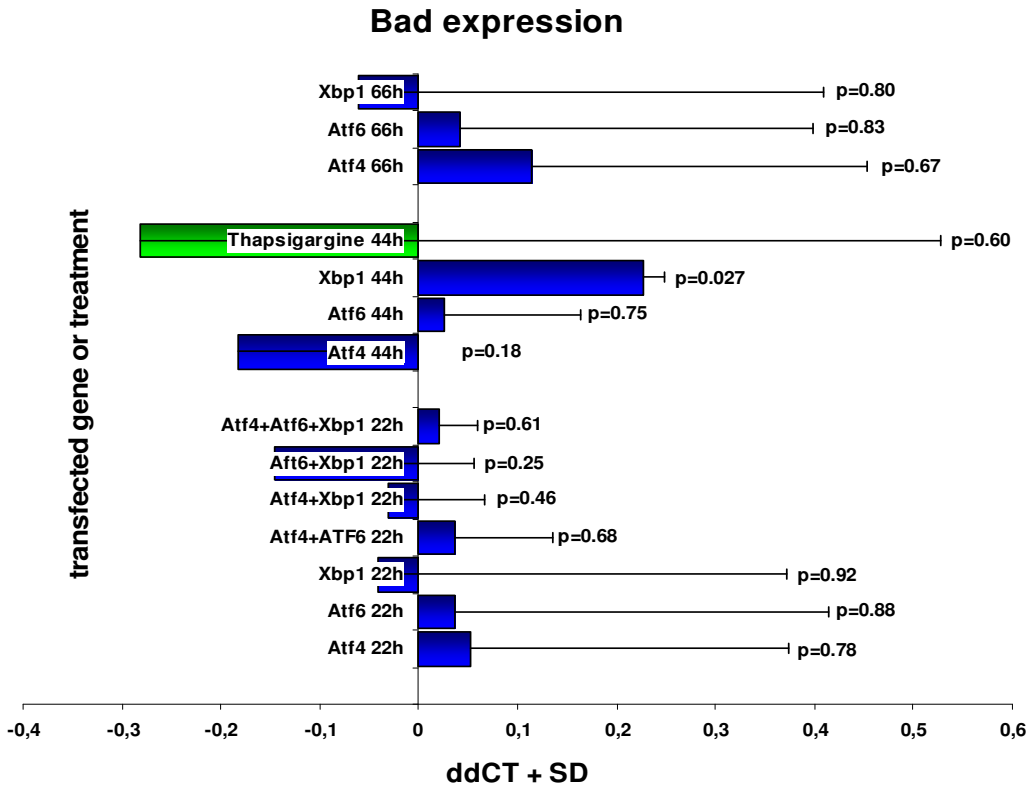
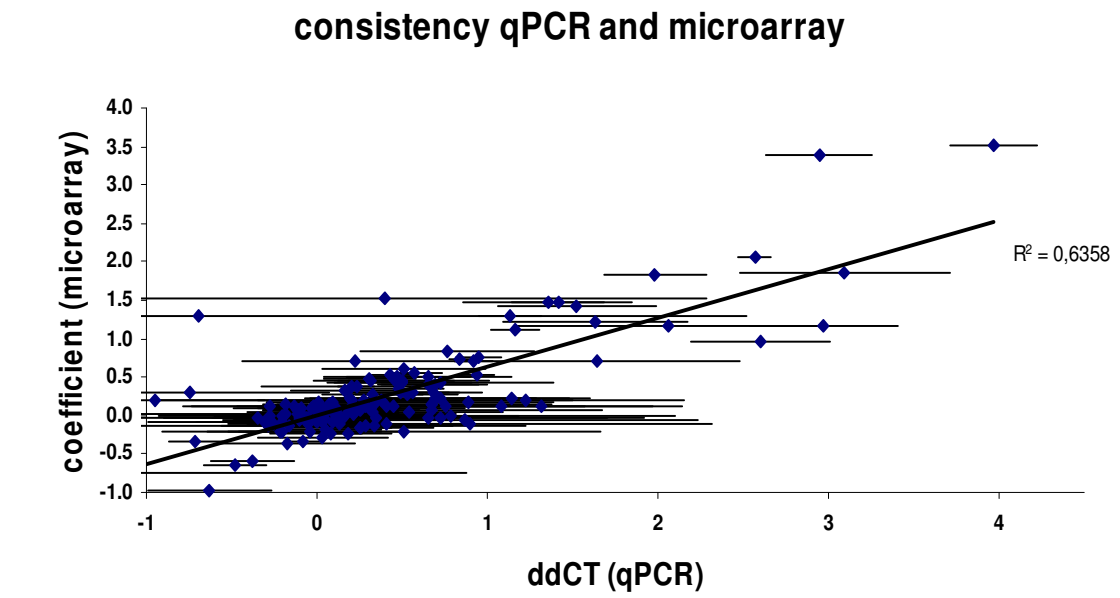


H

***Figure 18*: Verification of microarray results by qPCR (continued).**

Continue *Figure 18*:



I

***Figure 18*: Verification of microarray results by qPCR**. Changes in A) Bip, B) Grp94, C) Vcp, D) Ppib, E) Edem, F) Atf3, G) Nfe2l2, H) Bad as a result of single or combined over-expression of Atf4, Atf6 and Xbp1 (blue bars) and TG-treatment (green bars). The gene expression changes are expressed as ddCT-values with standard deviation. I) The changes in gene expression by qPCR of the genes shown above plus Atf4, Chop, Dnajc10, Atg12, Chkb and Nf-y in qPCR (± standard deviation) are plotted against the changes as assessed by microarrays. A linear regression line and the corresponding indicator R^2 show the linear dependency of both values.

The qPCR data can be looked upon as a confirmation of the microarray results. *Figure 18 I* shows a good linear dependency between the lfc in the microarrays and the ddCT from the qPCR. Included were all results from the qPCRs mentioned in this thesis, except Atf6 and Xbp1, because the microarrays cannot identify spliced Xbp1, like the qPCR and the probes for Atf6 are not correct. Taken together the qPCRs (in addition to the microarray results) confirm the general functionality of the transgenes by their effect.

4.3.2 Effects of Atf4, Atf6 and Xbp1 on miRNA expression

MiRNA-arrays of MLE12 cells transfected with Atf4, Atf6 or Xbp1 revealed only a few candidates (adj. $p < 0.05$) for regulated miRNAs and most of these candidates

showed rather small lfc (<0.5). The best candidates are miR-130b and miR148a (see *table 3*).

***Table 3*: Hit list for miRNAs regulated differentially by ATF4, ATF6 and XBP1.**

Hit list:

ProbeID	Symbol	ATF4 coefficients	ATF6 coefficients	Xbp1 coefficients	ATF4 adj.p	ATF6 adj.p	Xbp1 adj.p
A_54_P1266	mmu-miR-130b	0.05	0.57	1.27	0.999	0.004	0.000
A_54_P1265	mmu-miR-130b	0.06	0.23	0.61	0.999	0.930	0.001
A_54_P1274	mmu-miR-148a	-0.04	0.76	0.56	0.999	0.000	0.000
A_54_P1273	mmu-miR-148a	0.03	0.71	0.52	0.999	0.000	0.000
A_54_P1443	mmu-miR-210	0.04	0.08	0.37	0.999	0.999	0.007
A_54_P2334	mmu-miR-152	0.03	0.14	0.36	0.999	0.934	0.002
A_54_P2488	mmu-miR-29a	-0.01	0.05	0.31	0.999	0.999	0.055
A_54_P2333	mmu-miR-152	0.06	0.17	0.28	0.999	0.206	0.003
A_54_P1382	mmu-miR-148b	0.00	0.11	0.19	0.999	0.999	0.070
A_54_P3830	mmu-miR-221	0.02	-0.03	0.18	0.999	0.999	0.015
A_54_P2348	mmu-miR-182	0.02	0.31	0.16	0.999	0.111	1.000
A_54_P1331	mmu-miR-96	-0.01	0.31	0.16	0.999	0.134	1.000
A_54_P2347	mmu-miR-182	0.07	0.28	0.06	0.999	0.165	1.000
A_54_P2445	mmu-miR-34b-5p	0.09	0.30	0.03	0.999	0.111	1.000
A_54_P2644	mmu-miR-222	-0.17	-0.15	-0.09	0.127	0.109	0.673
A_54_P2494	mmu-miR-92a	-0.14	-0.17	-0.20	0.999	0.165	0.036
A_54_P3503	mmu-miR-17*	-0.07	-0.09	-0.25	0.999	0.999	0.006
A_54_P00004755	mmu-miR-2135_v15.0	-0.20	-0.43	-0.30	0.999	0.162	0.786
A_54_P00004734	mmu-miR-2138_v15.0	-0.22	-0.39	-0.33	0.999	0.144	0.312
A_54_P1980	mmu-miR-689_v15.0	-0.20	-0.32	-0.34	0.999	0.072	0.015
A_54_P3447	mmu-miR-21*	-0.03	-0.13	-0.36	0.999	0.930	0.001
A_54_P4365	mmu-miR-101a	-0.13	-0.23	-0.36	0.999	0.498	0.023

Depicted are such miRNAs with an adjusted p (adj.p) $\leq$0.2 for at least one of the transfections (ATF4, ATF6 and Xbp1). For some miRNAs (Symbol) more than one probe (ProbeID) matched these criteria. Significant changes are highlighted in blue.

Nevertheless, the overall regulation of miRNAs by Atf4, Atf6 and Xbp1 was quite similar, as the correlation analysis reveals (see *table 4*).

***Table 4*: Correlation of miRNA expression in ATF4, ATF6 and XBP1 transfected MLE12 cells**

	correlation	95 percent confidence interval		regression line	
	Pearson´s r	lower	upper	intercept	slope
Xbp1 vs. ATF4	0.43	0.39	0.46	0.00	0.58
Xbp1 vs. ATF6	0.61	0.58	0.64	0.01	0.69
ATF4 vs. ATF 6	0.54	0.51	0.58	0.01	0.45
correlation without extreme values(|coef| > 0.2)					
Xbp1 vs. ATF4	0.42	0.38	0.46	0.01	0.43
Xbp1 vs. ATF6	0.55	0.52	0.58	0.01	0.52
ATF4 vs. ATF 6	0.49	0.46	0.53	0.01	0.57

The table lists indicators (Pearson's r + upper and lower confidence interval) for a correlation between the miRNA expression changes as a result of over-expression of Atf4, Atf6 and Xbp1. The depicted (positive) slope of the regression line indicates that the changes compared are not zero. The lower part of the table does not include the miRNAs with "stronger" regulation (coefficient >0.2).

4.3.3 Effects of Atf4, Atf6 and Xbp1 on mRNA splicing

Splicing of target mRNAs was assessed 24 h after over-expression of Atf4, Atf6 and Xbp1(s) respectively in 400K-Exon arrays. Genes where the lfc of an individual probe was at least three different from the mean were preselected as candidates for alternative splicing (52 after Atf4-transfection, 64 after Atf6 transfection and 108 after Xbp1(s) transfection). There was a big overlap of the candidates of alternative splicing between the three transfected transgenes. For the purpose of this thesis only a more restricted list of "top candidates" shall be presented (see *table 5*). Only such genes, where the outlier-probe had a log2 of the signal intensity >5 (which is an argument for robust expression of the candidate gene) and the 63 % confidence interval did not touch the zero-line are included.

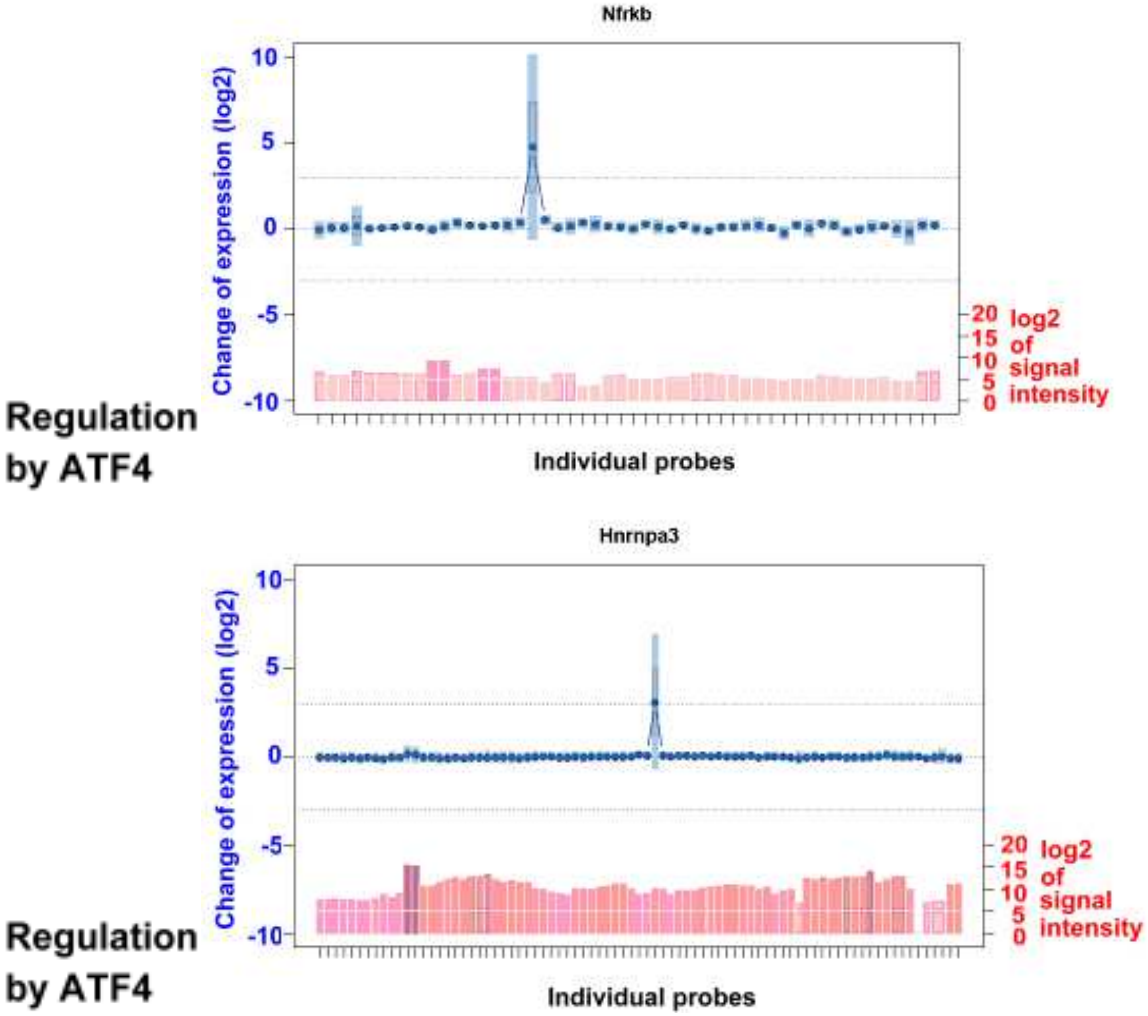
***Table 5*: Top candidate genes for alternative splicing initiated by ATF4, ATF6 or XBP1 over-expression**

Top candidate genes, alternatively spliced in response to overexpression of			Number and direction of the outlyer regions
Atf4	Atf6	Xbp1	
	Derl3		2x up
	Cep290		up
		Sec24a	up
Zw10	Zw10	Zw10	down
	Gtf3c3		up
Ncoa2	Ncoa2		down
		Rps6ka1	up
		Zfp653	up
Nfrkb			up
Hnrnpa3			up
		BC085271	down
	Cotl1		up
	Pgam5		down
		Kif3a	down
		Porcn	down
Cd3e			up
Fam102a			2x up
Fbln2			2x up
Erbb2ip	Erbb2ip	Erbb2ip	down
	Svil		up
		Pus3	down
4932438A13Rik	4932438A13Rik	4932438A13Rik	2x down
		Cecr2	up
Uhrf1bp1l	Uhrf1bp1l	Uhrf1bp1l	down

Depicted are such genes with exons with an lfc of $\geq$ three (as compared to the mean of al exons of the gene) and were the log2 signal intensity of this exon was > 5 and the 63 % confidence interval did not touch the zero line. The right column indicates whether the respective exon was up or down-regulated (as compared to the other exons of the gene) and if more than one exon was affected (if two neighbour probes were regulated in the same direction they were counted as one exon here).

Some potential functions of those candidates shall be named here: The first four candidates can directly be linked to the topic of ER-Stress. Derl3 is involved in the ERAD. Cep290 was described to be an activator of Atf4-mediated transcription. Sec24a and Zw10 are involved in ER to Golgi – trafficking. Gtf3c3, Ncoa2, Rps6ka1, Zfp653 and Nfrkb are involved in regulation of transcription and Hnrnpa3 is involved in nuclear mRNA splicing. The results for those genes are depicted here in more detail (see *figure 19*, following pages). In case of the top-candidates

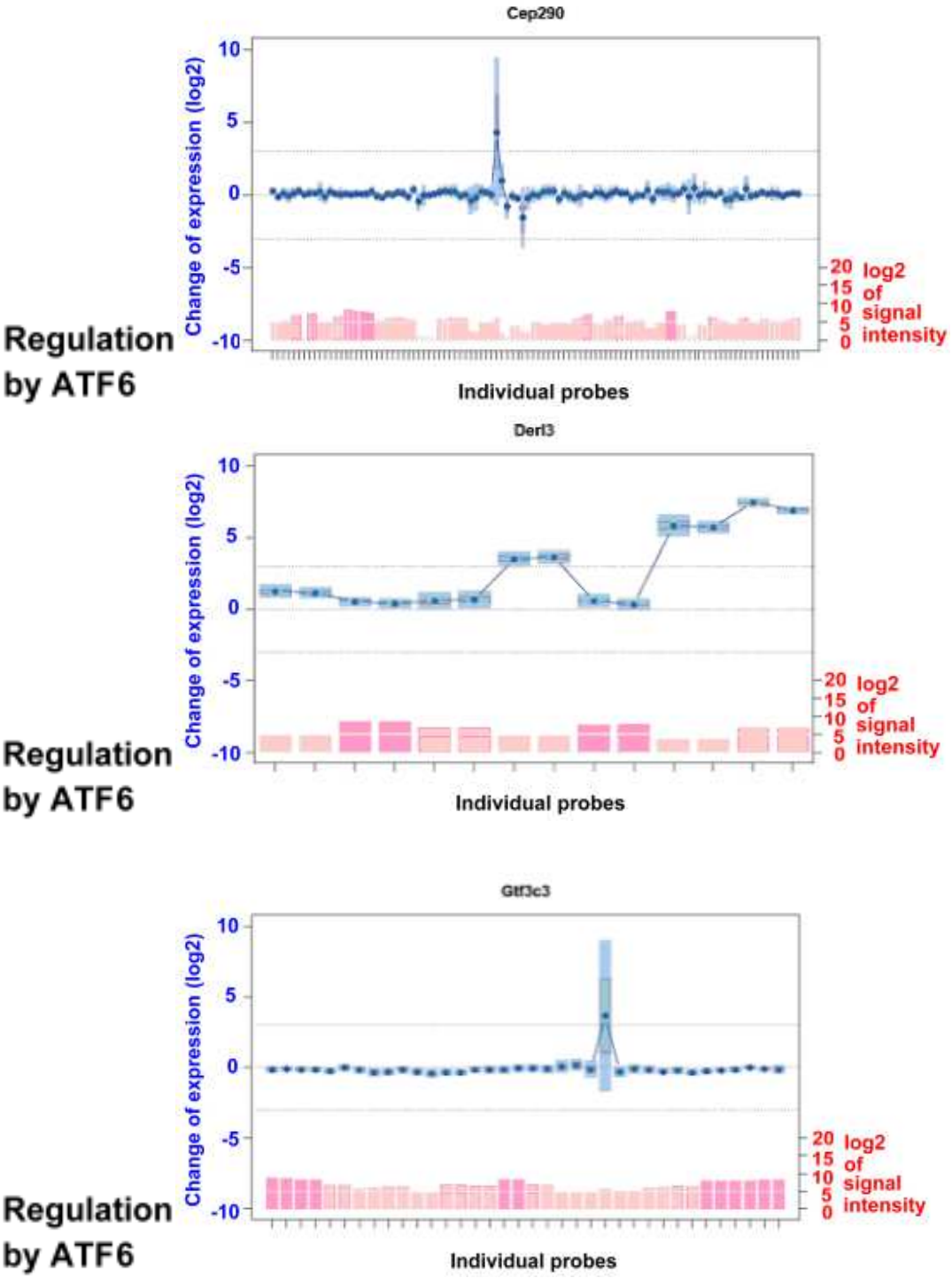
which were found after transfection with different transgenes (also those not shown in detail in *figure 19*), the same outlier- probes were regulated in the same direction.



A) Differential regulation of Nfrkb and Hnrnpa3- Exons by Atf4

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection (continued).**

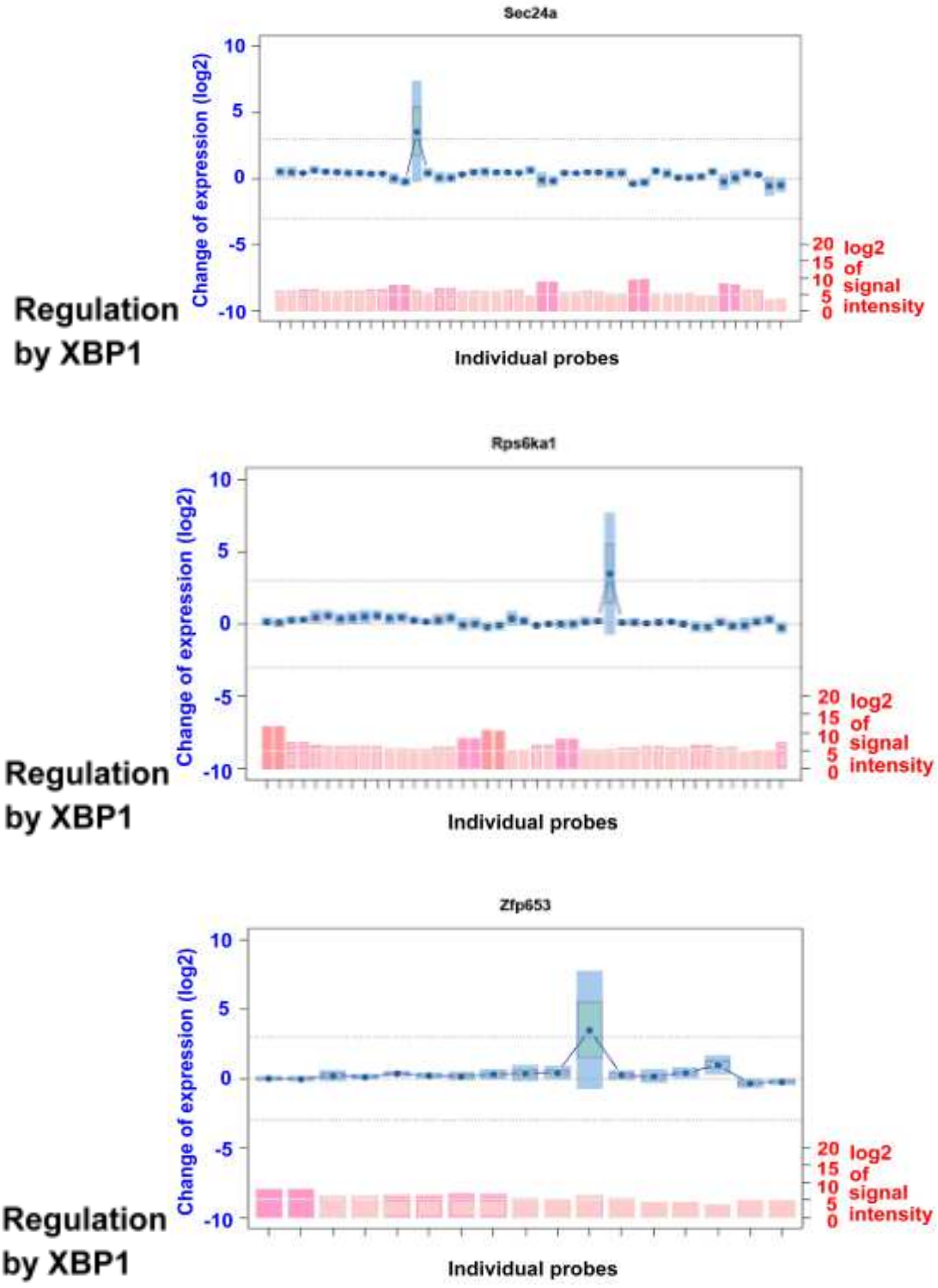
Continue *figure 19*:



B) Differential regulation of Cep290, Der3 and Gtf3c3- Exons by Atf6

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection (continued).**

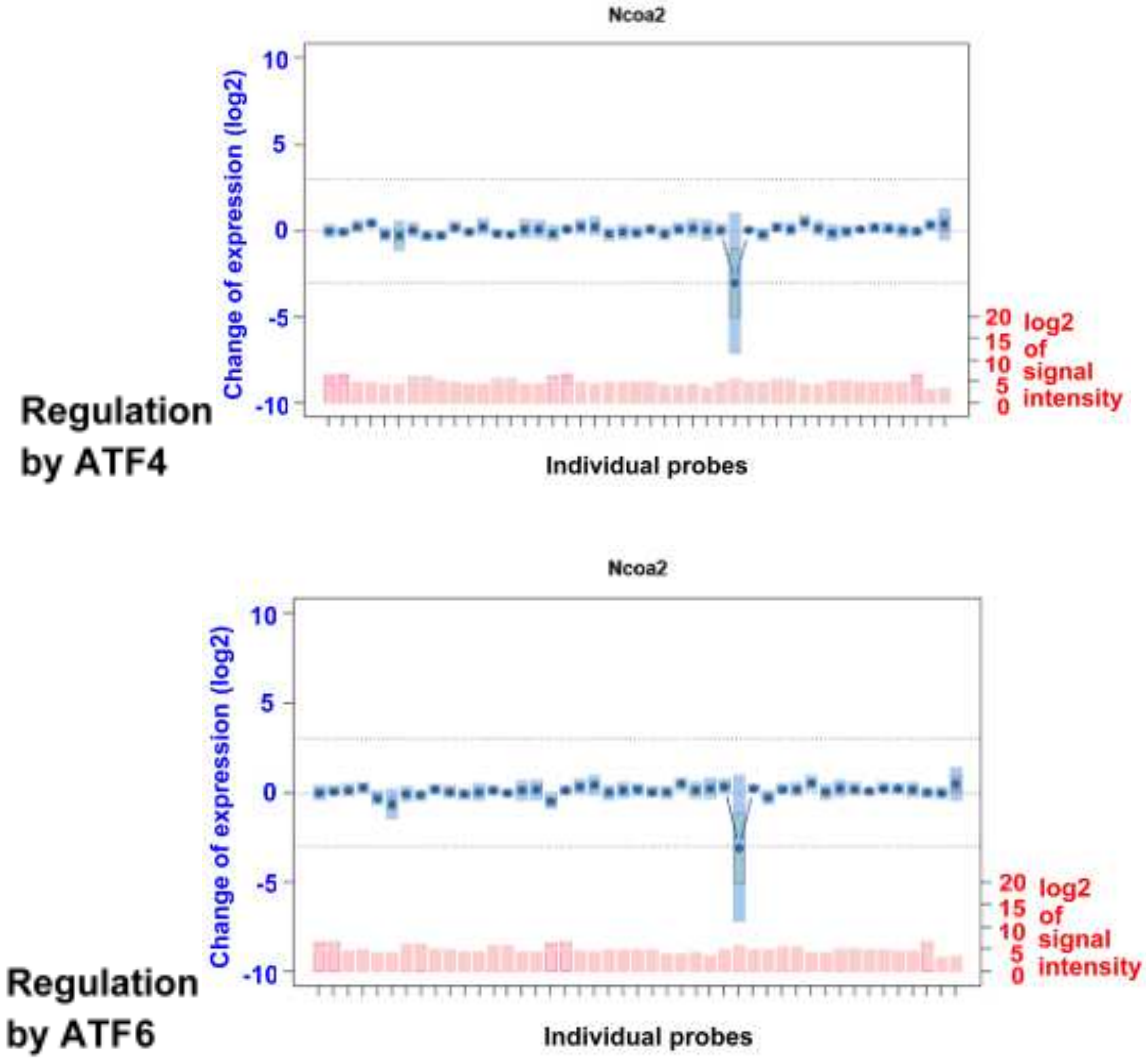
Continue *figure 19*:



C) Differential regulation of Sec24a, Rps6ka1 and Zfp653-Exons by Xbp1

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection (continued).**

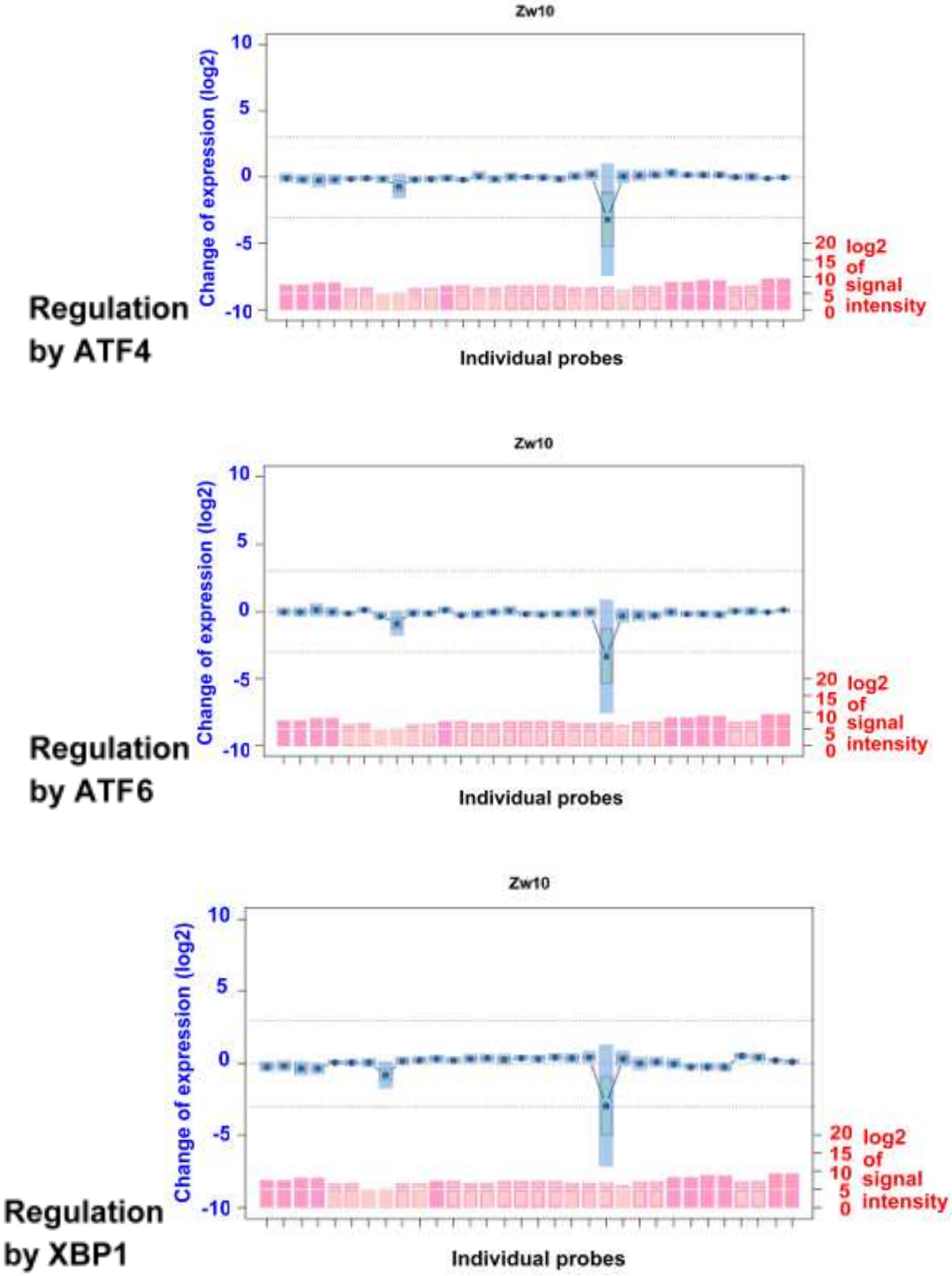
Continue *figure 19*:



D) Uniform regulation of Ncoa2- Exons by ATF4 and Atf6

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection (continued).**

Continue *figure 19*:



E) Uniform regulation of Zw10- Exons by ATF4, Atf6 and Xbp1

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection (continued).**

***Figure 19*: Selected top candidates for alternative splicing as a result of Atf4, Atf6 or Xbp1-transfection.** Each transgene was leading to an individual set of top candidates for alternative splicing (A, B and C) but there were also candidates which were regulated uniformly by more than one of the transgenes (D+E). Red (scale on the right hand): log2 of the signal intensity; Blue (scale on the left hand): change of expression of individual probes in response to the transfection with Atf4, Atf6 and Xbp1 (relative to the mean) with 63 and 95 % confidence interval. On the X-Axis the regulation of the individual probes (representing exons or parts of them) are depicted.

4.4 Specific cellular responses to over-expression of ATF4, ATF6 and XBP1 *in vitro*

4.4.1 CHOP induction by Atf4, Atf6, Xbp1 and the ER-stress inducers Brefeldin A (BFA) and Thapsigargin (TG)

ER-stress is supposed to cause CHOP induction via the three transcription factors ATF4, ATF6 and XBP1 (Szegezdi *et al*. 2006). This could not be observed in the alveolar epithelial cell line MLE12. On protein level, CHOP induction could only be observed after treatment with the ER-stress inducing agent TG (see *figure 20*).

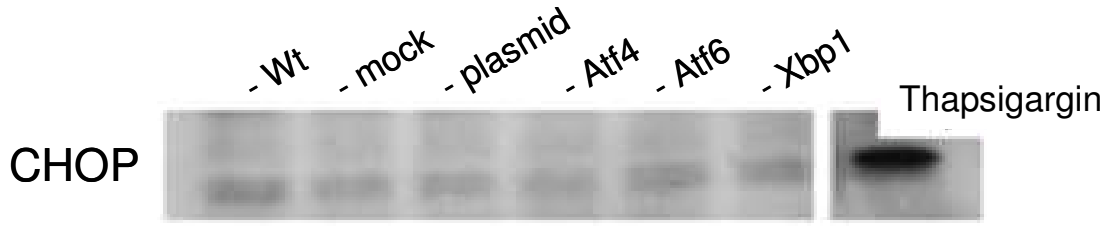


***Figure 20*: CHOP-protein expression in Atf4, Atf6 and Xbp1 transfected MLE12-cells.** The controls are WT (untreated cells), mock (treated with transfection reagent without DNA), plasmid (control transfection with the plasmid pIRES2-dsRed2) and TG (1 µM on WT-cells), the sample was loaded on the same gel, unrelated lanes are cut out. The incubation time between TG treatment / transfection and harvesting was 24 h.

The lack of CHOP-induction was not limited to the protein level. Even though a certain (in some cases significant) tendency of CHOP induction could be observed for the three transgenes, the effect was minimal as compared to the CHOP-induction caused by TG.
Also the combined transfection of these factors did not lead to a robust induction of Chop (see *figure 21*).

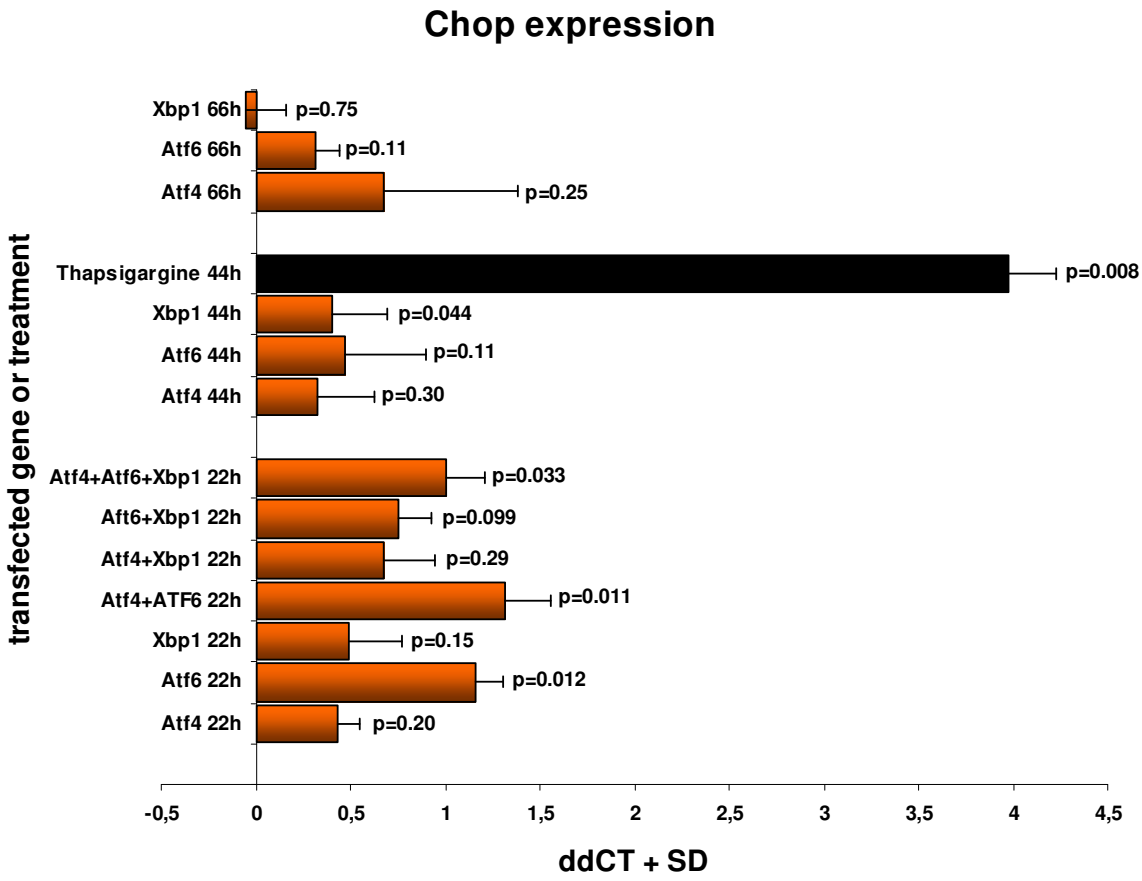
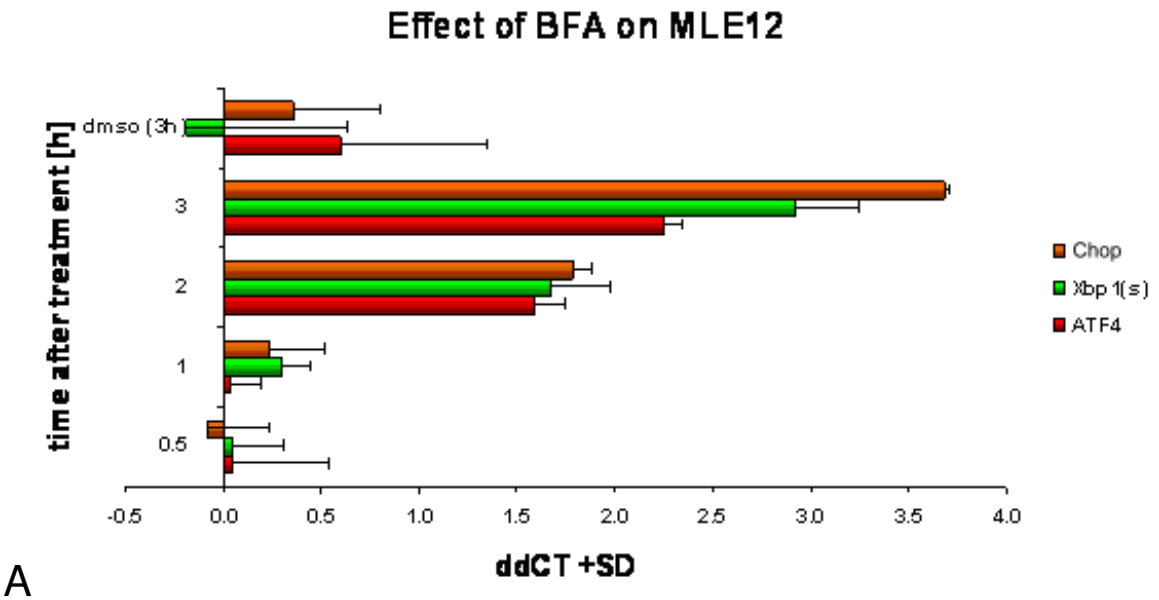


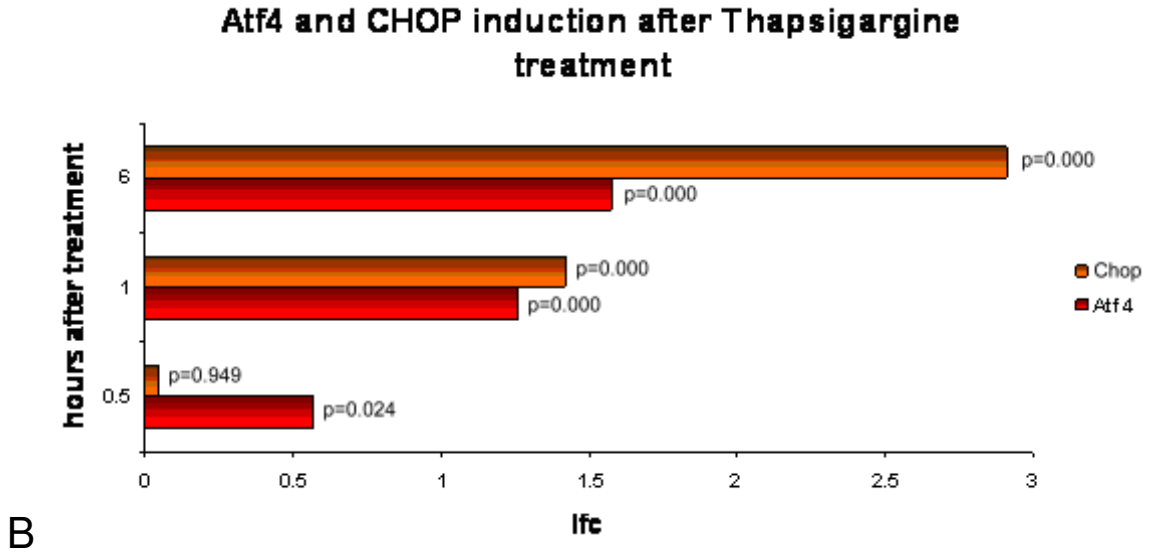
***Figure 21*: Chop-mRNA expression in Atf4, Atf6 and Xbp1 transfected MLE12-cells.** A-C) MLE12 cells were transfected with the cell culture constructs for Atf4, Atf6 or Xbp1(s) (see 4.1.1) for 22h, 44 h or 66 h as indicated or with half the amount of a combination of two transfection mixes or one third of the three together (for 22 h). Transfections with the empty plasmid (pIRES2dsRed2) for the same durations served as a reference to calculate the ddCT. TG treatment (black bar) was done on top of 22 h empty vector transfection and another 22 h incubation, leading to the indicated 44 h. N=3.

To check whether this lack of response is limited to the MLE12 cell line, another alveolar epithelial cell line MLE15, and, a human keratinocyte cell line MEL188, were similarly transfected with Atf4, Atf6 and Xbp1. A robust Chop induction could not be observed in both systems (see *supplementary figure 1*).

In a separate experiment, MLE12 cells were treated with high doses of BFA (10 µg/ml). The expression of Atf4, spliced Xbp1 and Chop was monitored by qPCR 0.5, 1, 2 and 3 h after the treatment. The induction of these factors could be observed simultaneously after 2 h at similar levels. After 3 h the induction of Chop was surpassing the Atf4 and Xbp1 induction.



A



B

***Figure 22*: Time dependent induction of UPR-transcription factors and Chop after chemically induced ER-stress.** A) The mRNA-levels of Chop, Atf4 and Xbp1(s) from cells incubated with BFA for 1, 2 and 3 h were assessed by qPCR (control: DMSO treatment, reference for ddCT calculation: untreated cells). N=3. B) Expression changes of Chop and Atf4 as assessed by microarrays, 0.5 h, 1 h and 6 h after TG-treatment (n=4 for treated cells and 12 for controls).

Treatment with 1µM TG (as assessed in microarrays) was leading to a robust induction of Chop and Atf4 after 1 h. After 6 h the induction of Chop was clearly stronger. A minor induction of Atf4 was already observed 0.5 h after transfection.

4.4.2 Atf4 and Atf6 knockdown is not sufficient to block CHOP-induction by TG and BFA

If Atf4 and Atf6 were required for Chop induction, a knockdown of these factors should reduce the inducibility of Chop by ER-stress generating agents. MLE12 cells were transfected with siRNAs against Atf4 and Atf6 and the reduction of their mRNA-levels was confirmed by qPCR (4.1.5). The base line level of Chop (as compared to NT-siRNA transfection) was moderately but significantly decreased in response to Atf4 and Atf4+Atf6 knockdown. However, a double knockdown of Atf4+Atf6 did not result in a reduction of Chop, induced by treatment for 2 h with BFA or TG.

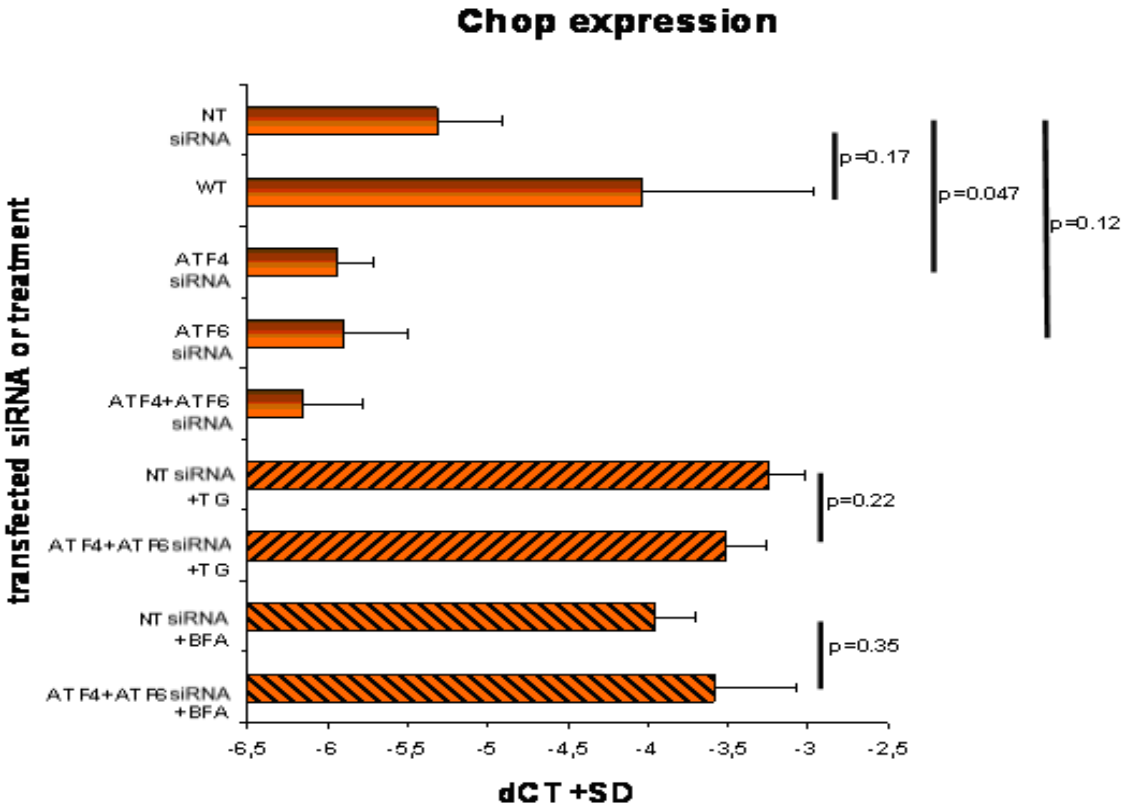
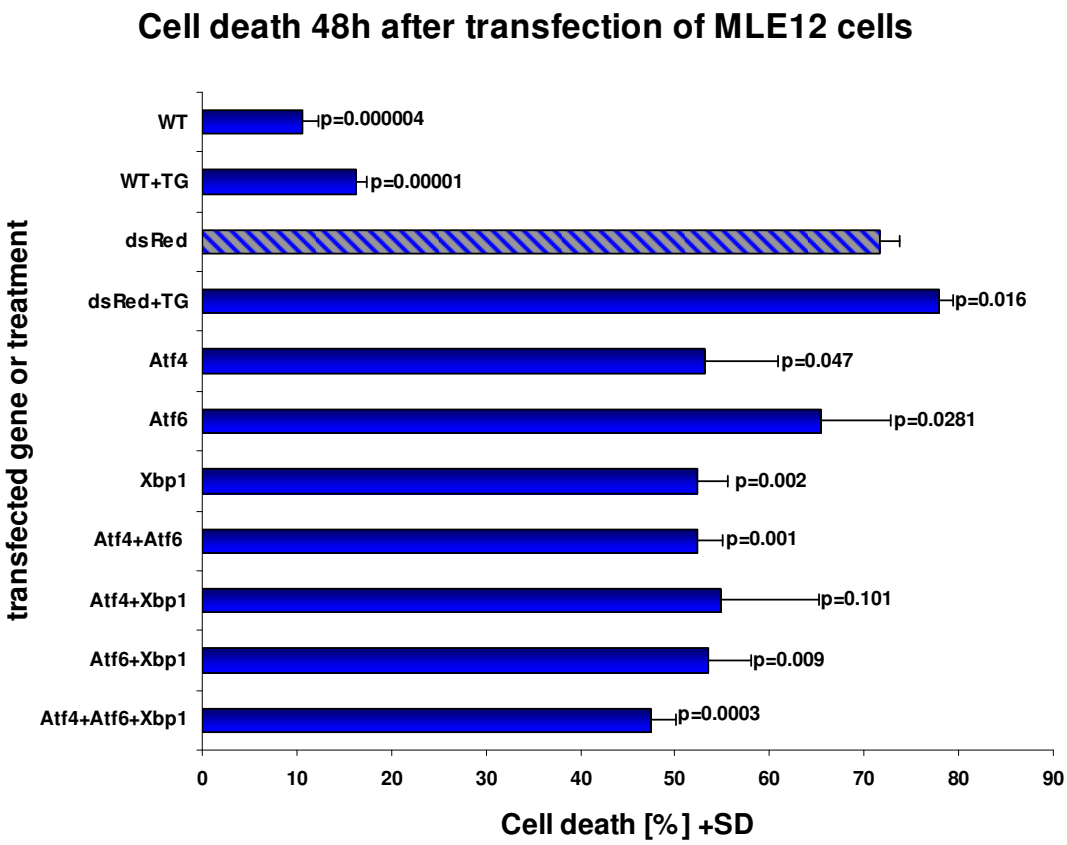


***Figure 23*: Effects of Atf4 / Atf6 knockdown on induced and non-induced Chop-expression.** MLE12 cells were transfected with siRNA against ATF4 or ATF6 or a combination of both. NT (non-targeting siRNA) served as a reference, WT (untreated) as an additional control (massive bars). Treatment with TG or BFA was held up for 2 h and started after a preincubation either with NT or Atf4 / Atf6 siRNA combitransfection (dashed bars). The harvested and reverse transcribed RNA was subject to qPCR for Chop. N=4.

4.4.3 Over-expression of Atf4, Atf6 and Xbp1 does not induce epithelial cell death *in vitro*

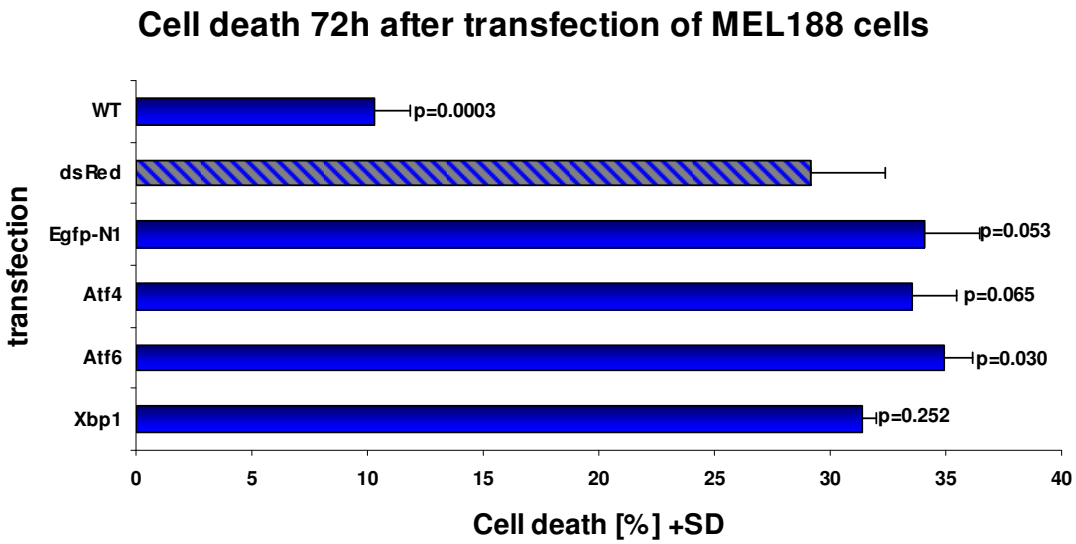
Atf4, Atf6 and Xbp1 are supposed to induce genes which help cells to overcome ER-stress. On the other hand they were described to induce Chop, which leads to apoptosis. To assess whether cell death was the result of the expression of these transgenes, lactate dehydrogenase (LDH)-assays were performed. 48 h after transfection, a high level of cell death could be observed as a result of the control transfection. None of the transgenes or their combination, but only treatment with TG, increased the cell death caused by the transfection. On the opposite, the effect of the transgenes was significantly beneficial (except for the combination of Atf4 and Xbp1 where only a tendency was encountered) (see *figure 24 A*). The experiment was repeated with the triple amount of DNA / Lipofectamine mix in the transfection. Thereby the cell death was further increased but the transgene effects were very similar (Data not shown).



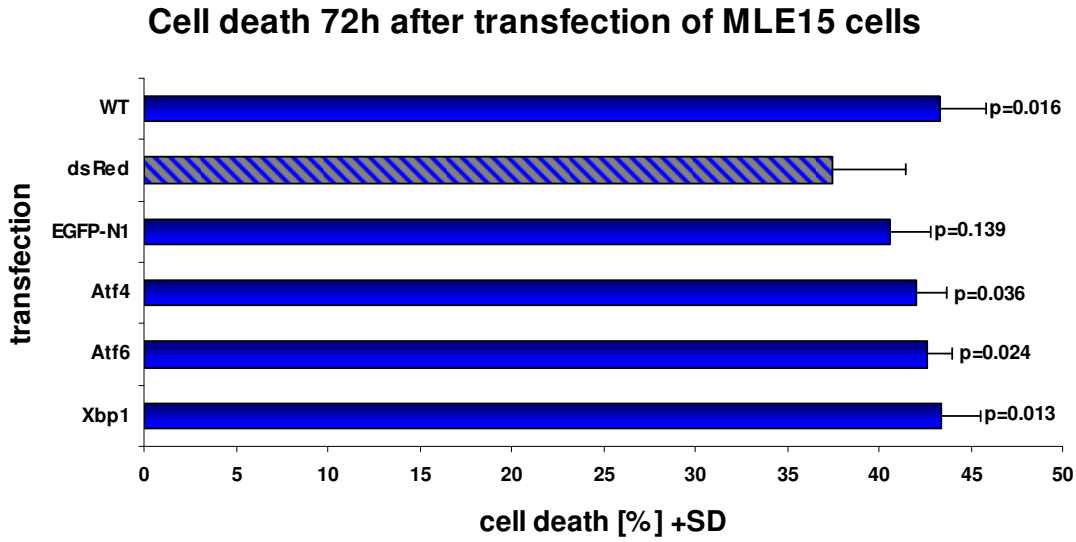
A

***Figure 24*: Effects of UPR-transcription factors on cell death in different cell types (continued).**

Continue *figure 24*:



B



C

***Figure 24*: Effects of UPR-transcription factors on cell death in different cell types.** Cell death of cells transfected with Atf4, Atf6 or Xbp1 (or combinations of them) was assessed by LDH assay. Transfection with the empty plasmid pIRES2dsRed2 (dsRed, dashed line) serves as the reference against which the p-values were calculated. MLE12 cells A) (n=3) were transfected with one, two or three of the transgenes. WT (untransfected) cells and cells treated with TG for 24 h (either WT of dsRed2-transfected cells) served as controls. The incubation time after transfection was 24 h. For MEL188 (n=6) B) and MLE15 (n=4) C) cells WT and EGFP-transfected cells served as a control (transfection for 72 h).

Transfection of MEL188 and MLE15-cells for 72h with these transgenes did not lead to robust changes in cell death as compared to empty vector control and transfection with an EGFP-plasmid (see *figure 24 B+C*).

To assess whether the effects of Atf4, Atf6 and Xbp1 could be driven into a maladaptive direction, the cells were challenged with peroxide and / or grown in starving medium after the transfection. The three challenges all caused a significant increase in cell death (about 5 %). But the transgenes did not have a robust, negative impact under these stress conditions (see *figure 25*).

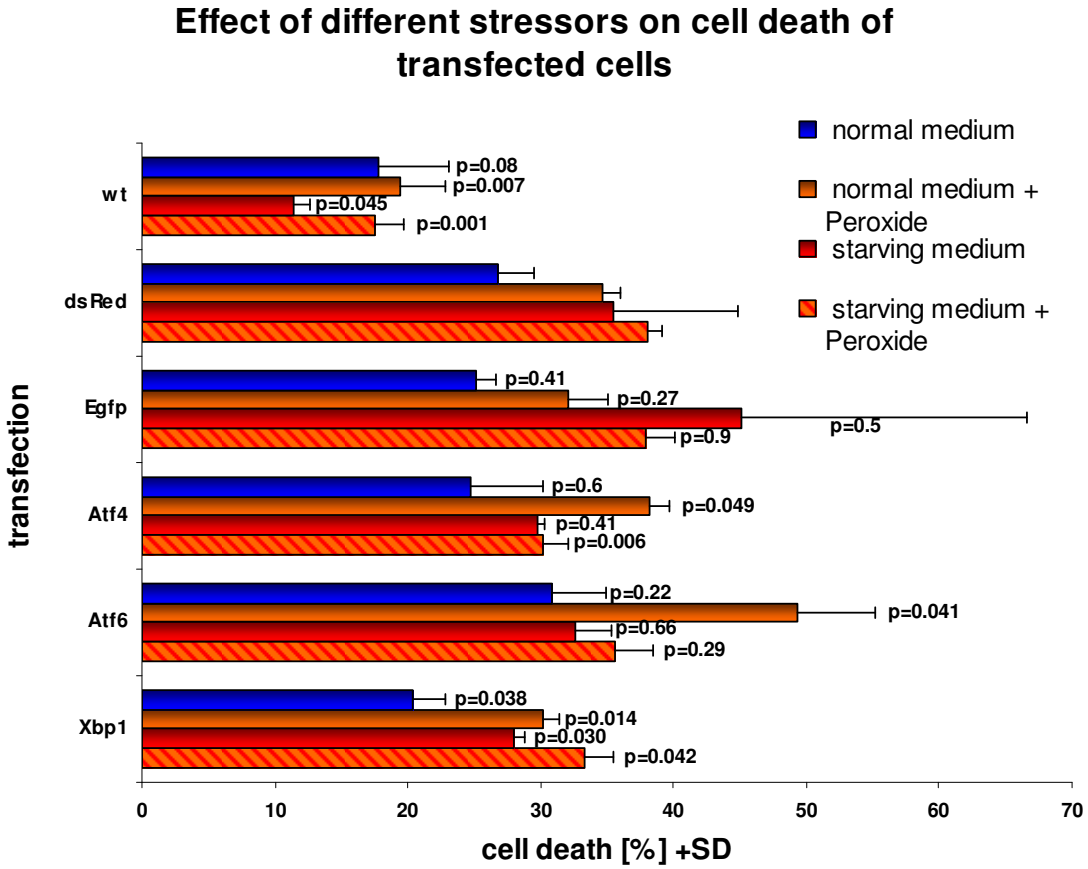
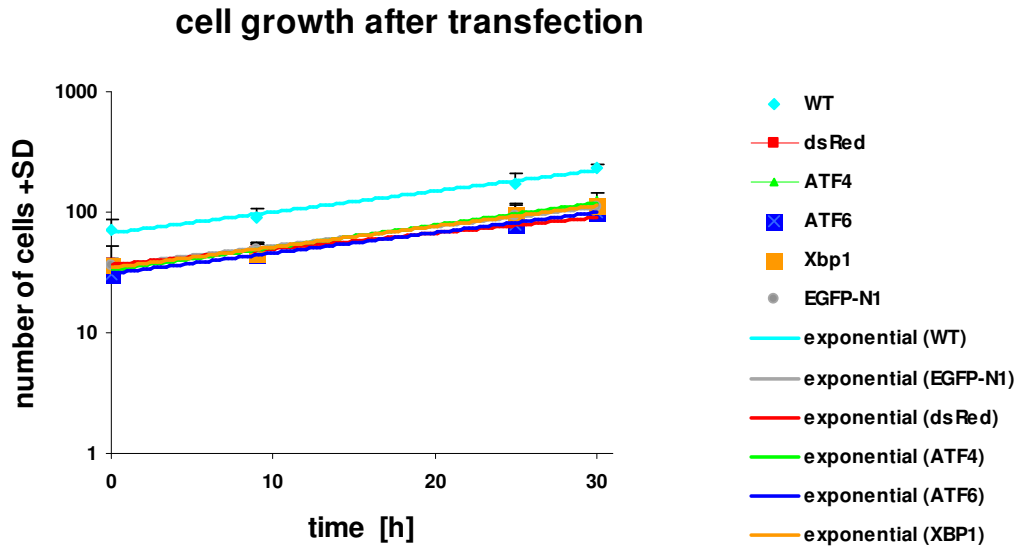


***Figure 25*: Effects of ATF4, ATF6 and XBP1 on stressed MLE12-cells.** Cell death of transfected and untransfected MLE12 cells with or without additional challenges was assessed by LDH assay. Transfection with the empty plasmid pIRES2dsRed2 (dsRed) serves as the reference against which the p-values were calculated. Egfp transfection serves as an additional control. The cells were incubated for 72 h after transfection. Treatment with peroxide and / or starving medium started 24 h after transfection. N=3.

4.4.4 Influence of Atf4, Atf6 and Xbp1 on cell growth

Cell growth in response to transfection with Atf4, Atf6 and Xbp1 was assessed as described in the methods part (see 3.4). It was compared to WT-cells, empty vector and EGFP-transfected cells. The transfection as such strongly reduced the cell number at the beginning of the observed growth phase but only caused a minor decrease in the slope of the growth curve as compared with the empty vector control. No other significant changes in the growth rates were observed (see *figure 26*).



	mean slope regression line	p= (for difference to dsRed)
WT1	0.058	0.045
dsRed-1	0.044	1.000
ATF4-1	0.062	0.069
ATF6-1	0.057	0.371
Xbp1-1	0.057	0.059
EGFP-N1 1	0.050	0.315

***Figure 26*: Effects of ATF4, ATF6 and XBP1 on cell growth.** MLE12-cells were transfected with Atf4, Atf6 or Xbp1 or dsRed2 (reference) or left unchallenged (WT). After plating on a fresh cell culture dish, the cells were counted at four time points. The cell numbers are depicted in the upper part of the diagram. The slopes of the regression lines (logarithmic scale) from three independent transfections are compared in the table below. P-values refer to the differences of the slope of regression lines from those of the reference transfections.

5. DISCUSSION

The purpose of this research project was to further elucidate signalling pathways and cellular consequences of endoplasmic reticulum stress in alveolar Type II (AECII) cells *in vivo* and alveolar epithelial cell lines (MLE12, MLE15) *in vitro*. The focus was on ATF4, ATF6 and XBP1 induced signalling, as these factors represent the three branches of the unfolded protein response (UPR). The underlying hypothesis was that, when over-expressed, these factors would, amongst others, induce the pro-apoptotic mediator CHOP and subsequently lead to apoptosis of the affected cells. *In vivo* this would result in chronic alveolar epithelial injury and, finally, initiation of an aberrant repair process, leading to lung fibrosis. This mechanism was proposed as a trigger of the fatal lung disease IPF.

5.1 Considerations about the model systems

As a substitute for primary isolated AECII, mainly MLE12 cells were used. For some control purposes MLE15 cells were utilized. Both cell lines are monoclonal and derived from murine lung tumors (generated in mice, harbouring the viral oncogene simian virus 40 under transcriptional control of a promoter region from human SP-C) and both share features of their progenitor cells (AECII). However, the two lines differ in tumorigenicity, expression and processing of surfactant proteins. In MLE12 cells, secretion of mature SP-C but not SP-B was detected. Secretion of phospholipids from MLE12 cells in response to ATP was limited to earlier passages. MLE12 cells do not express SP-A and do not contain lamellar bodies (Wikenheiser *et al*. 1993). Isolation of primary AECII is expensive and time consuming. As isolated AECII (from murine origin) do not proliferate, the availability of these cells is limited. In addition the purity of the cultures is difficult to guaranty (Corti *et al.* 1996; Roper 2003) and once taken in culture these cells differentiate and loose features of AECII. The view that this process is a trans-differentiation to an AECI-like phenotype is not supported by transcriptome profiling (Gonzalez *et al*. 2005). Also the efficiency of transfection is typically low for murine AECII. In contrast to that, optimal transfection efficiencies could be achieved with all utilized cell culture lines in this work and expression of the transgenes was high (*figure 7*).

For all these reasons, MLE cell lines are commonly used in lung research (Lozon, Altemeier 2009; Zhong *et al*. 2010; Yoshida *et al*. 2010; Sisson *et al*. 2010;

Mulugeta *et al*. 2007) and were also considered a sufficient substitute for AECII in my *in vitro* studies.

MEL188 cells are a human skin derived cell line which was used as a non-AECII-derived but also secretory control cell line, which shows distinct morphological similarities, such as lamellar bodies due to synthesis and secretion of lipids.

In spite of the many advantages of MLE – cell lines it has to be taken into account that they are not identical to AECII. The possibility can not be excluded that they react different on ER-stress signalling and especially differ in the mechanisms of apoptosis induction. It can also not be excluded that AECII behave different in the context of an intact lung as compared to cell culture systems. Consequences of transgene expression on the tissue and organ surrounding of the directly affected cells can only be investigated *in vivo* anyways.

Another potential problem is the transfection with the additional markers dsRed2 (*in vitro*) and luciferase (*in vivo*). Over-expression of transgenes can generally lead to ER-stress, as demonstrated for the lens by (Reneker *et al*. 2011). It therefore has to be considered, that the results do not reflect the effect of ATF4, ATF6 and XBP1 respectively in naive cells but likely in such with a certain amount of ER-stress. *In vitro* there was increased early cell death caused by transfection (*figure 24+25*). However, there was no detectable CHOP induction caused by the transfection (*figure 20*) and the amount of cleaved ATF6 was even decreased (*figure 7 E*). The observed cell death was rather an immediate effect of the transfection than ER-stress induced apoptosis.

We must also consider the way the transfected transgenes normally act and how they are integrated in the respective UPR-pathways. ATF4 (translation) is activated as a direct result of eIF2α phosphorylation and then activates target gene expression. Some of these targets are other transcription factors. However, it is still possible that other transcription factors are also directly activated by eIF2α phosphorylation (reviewed by Ron 2002). ATF4 is required for eIF2α dephosphorylation, involved in reactivation of protein synthesis and BIP-protein synthesis after TG treatment. ATF4 directly binds the Gadd34 promoter at its ATF-site and activates it. As discussed elsewhere the ATF4 downstream target ATF3 is also involved in Gadd34 regulation. Gadd34 (This study: Myd116: ATF6 22h: -0.91; TG 22h: +2.40; TG 6h: +2.39)[4] dephosphorylates eIF2α (Ma, Hendershot

[4] The values are data from my microarrays depicting the regulation of the indicated gene by ATF4, ATF6, XBP1 or TG after the indicated time-points after treatment with the reagent or after transfection. The format is subsequently generally used to repeat microarray results. Generally the gene name from the micrarrays is

2003). ATF4 does not necessarily present the complete pathway and the negative feedback loop can potentially shut down the endogenous ATF4 production and reduce CHOP-induction (Novoa *et al*. 2001).

ATF6 activation includes its transport from the ER to the Golgi. Other folded proteins are transported together with ATF6, but there is no leak of unfolded proteins in that pathway as they are effectively retained in the ER (Nadanaka *et al*. 2004). Reduced C-terminal glycosylation of p90-ATF6 on N-linked glycosylation sites has been identified as a factor for ATF6 activation (Hong *et al*. 2004). However, the effects of over-expression of the ATF6-N-terminal domain should not be influenced by a reduced glycosylation. These features are therefore not of any concern in my system.

IRE1 is not only responsible for XBP1-splicing, but also cleaves mRNA-targeted to the ER and thereby reduces the ER-load (reviewed by Eizirik, Cnop 2010). In light of this observation it is the more surprising that over-expression of XBP1(s) without induction of this protective mechanism did not induce apoptosis in my system.

On the other hand, XBP-1 production is essential for the proper function of specialized secretory organs, such as liver, pancreas, salivary gland and plasma B-lymphocytes (Zhang *et al*. 2005), in which a high rate of protein synthesis constitutes an endogenous source of stress (reviewed by Hetz, Glimcher 2008). It is therefore per se unlikely that pro-apoptotic signalling in these cells should depend on increased XBP1(s) levels.

CHOP action is not under all circumstances pro-apoptotic. Posttranslational modifications of CHOP (Halterman *et al*. 2010) and the influence of protective factors against the downstream effects of CHOP (Gotoh *et al*. 2004) were indicated to play a role here (also see 5.3).

The house mouse has several advantages that make it an almost perfect model organism for geneticists. Mice are easy to keep and breed all year round, with a short generation time (10–12 weeks). Unlike many other species, inbreeding is well tolerated (Guenet, Bonhomme 2003). Due to large litters and prominent pronuclei which facilitate microinjection of DNA, the inbred line FVB/N was especially recommended for generation of transgenic animals (Taketo *et al*. 1991). The generation of transgenic mice with inducible and AECII specific over-expression of ATF4, ATF6, XBP1 and additionally CHOP was a necessary,

used in this context, even if a more common name is used in the text. So in this example Myd116 is another name for Gadd34. For convenience this data is also summarized in S*upplementary table 7*.

but not yet fully completed, part of this work[5]. To achieve specificity and inducibility the TET-on system was utilized (for explanations see 4.1.2 and 3.5 for technical details).
ER-stress responses including CHOP induction are age dependent. Ageing generally decreases levels of ER chaperones but increases CHOP levels (reviews by Naidoo 2009 and van der Vlies *et al*. 2003). CHOP-induction and apoptosis is increased in aged hepatocytes (reviewed by Puzianowska-Kuznicka, Kuznicki 2009). In another system CHOP levels were significantly lower in the aged than the adult brain (Sandhir, Berman 2010). Cell culture cells were therefore used in defined passage numbers and the age of mice going for transgene induction was also identical (see 3.1.4). It was not assessed weather older MLE12 cells reacted differently on the UPR-inducer transfections.

5.2 Broad Effects of ER-stress and the UPR

The current understanding of the UPR is that it leads to a general translational inhibition, enhances the folding capacity of the ER and up-regulates factors of the ERAD. In case of maladaptive ER-stress, it can also induce apoptosis. These components of the UPR are well confirmed by numerous publications and were extensively reviewed (Kapoor, Sanyal 2009; Ron 2002; Schröder, Kaufman 2005; Szegezdi *et al*. 2006; Xu *et al*. 2005; Zhao, Ackerman 2006). Examples for expected targets are Bip, Grp94 (chaperones), VCP (integral component of ERAD and cellular stress pathways induced by the UPR) (Vij *et al*. 2006), Ppib, Edem1 and Atf3 (qPCR results in *figure 18*). Of note ATF4, even though influencing folding, sorting and degradation pathways (*supplementary table 1*) regulated none of these factors.

If we look a bit closer, we see that the response to ER-stress can go far beyond those canonical effects. Some authors also already highlighted much broader effects, like Acosta-Alvear as “unexpected pathways” (Acosta-Alvear *et al*. 2007). In my work 55 % of all tested pathways (KEGG-Pathways which exist in the mouse) and 32 % of all probes were significantly regulated by Atf4, Atf6 or Xbp1 over-expression or TG-treatment (a bit more if we also consider additional candidates from microarrays with short term TG-treatment). Some known non-canonical pathways were identified in my microarrays. As an example ER-stress has been indicated to play a role in dysfunction of gap junctions by

[5] See the Chapter 5.5 I*n vivo* studies

down-regulating connexin 43 (Huang *et al*. 2009). This was also observed in my microarrays (This study: Gja1: TG 6h: -1.78). Consistently, the gap junction pathway was regulated by TG (22h) and ATF4, ATF6 and Xbp1 (see *supplementary table1*). Another example is Trib3, which is up-regulated in neuronal cell death scenarios (including TG induced cell death) and was identified as a binding partner of ATF4. It is able to activate CRE-dependent transcriptional activity of ATF4, without altering the degradation of ATF4 (Ord, Ord 2003). Trib3 is robustly up-regulated by ATF4 and TG (This study: Trib3: ATF4 22h: +1.05; TG 22h: +3.1; TG 6h: +3.69) and moderately by ATF6 (This study: Trib3: 22 h: +0.50). One well known function of ATF6 is the induction of Xbp1-mRNA (Yoshida *et al*. 2001). This cannot be observed in the qPCR because the primers used, specifically bind the spliced form of XBP1. However, the microarrays revealed a robust Xbp1- induction (This study: ATF6 22h: +1.84).

Other known pathways were not entirely regulated the way the literature suggests. ATF4 can bind Nrf2. The dimer is able to induce the heme oxygenase1 (This study: Hmox1: ATF6 22h: -0.39; TG 22h: +1.62) gene and further up-regulate ATF4 (He *et al*. 2001). Nfe2l2 (gene for NRF2) was not significantly regulated by single over-expression of ATF4, ATF6 of XBP1, but mildly down-regulated by any combination of those factors or TG (see *figure 18 G*).

Weak ER-stress was demonstrated to cause dedifferentiation and EMT in thyroid cells (Ulianich *et al*. 2008). In AECII, lower levels of ER-stress cause EMT, not apoptosis. This was demonstrated by down-regulation of E-cadherin (This study: Cdh1: TG22h: -1.35; TG 6h -1.14) and zonula occludens-1 (This study: Tjp1: ATF6 22h: -0.49) levels and up-regulation of α-smooth muscle actin (This study: Acta2: TG 22h: -0.61; TG 6h: -0.88) (Zhong *et al*. 2010). The robust down-regulation of Cdh1 by the strong apoptotic ER-stress of TG-treatment and the almost complete lack of regulation of these factors in response to ATF4, ATF6 and XBP1 over-expression observed in my system do not support this view.

ER-stress can also trigger autophagy (Yorimitsu *et al*. 2006). Atf4 was indicated to induce Atg12 and thereby also autophagy (reviewed by Hoyer-Hansen, Jaattela 2007). On the basis of a qPCR significant regulation of Atg12 was not observed in my work. However, on level of microarrays a minor effect of Xbp1 (This study: Atg12: XBP1 22h: +0.47) and more robustly TG (This study: Atg12: TG 22h +0.37, TG 1h: +0.45; TG 6h: +1.11) was observed.

Toll-like receptor signalling pathway was found to be regulated by ATF4, ATF6 and XBP1 in the microarray experiments but not by TG at any investigated time point (see *supplementary table 1*). One well described example is TLR2, which

was reported to be up-regulated in epithelial cells by ER-stress especially via ATF4 (Shimasaki *et al*. 2010). However, in MLE12-cells this is only partly true. Indeed, ATF4 up-regulated TLR2 (This study: Tlr2: ATF4 22 h: +0.73). But regulation by TG was limited to a very weak (This study: Tlr2: TG 1h: +0.54) up-regulation, which only occurred 1h after treatment and not sooner or later. Moreover ATF6 down-regulated TLR2 (This study: Tlr2: ATF6 22h: -0.64). The absence of robust TLR2 regulation by TG-treatment might therefore represent a combined effect of ATF4 and ATF6 signalling pathways.

These results are drawing an image of an extremely broad signalling in the context of ER-stress and the UPR, which is in no way limited to the canonical issues of the UPR and can differ from results generated in other models. On the first glance, the three UPR-transcription factors were regulating a common set of transcripts. Focussing specifically on the up-regulated genes, we found a much lower correlation between the effects of the transgenes, but a higher correlation with the TG-effect (see *figures 27 in 5.3 and 16+17 in 4.4.1*). This indicates, that the three factors are not just redundant systems but work together to induce the expression of all the genes which are required in the context of the UPR (and therefore all induced by TG). This view is supported by previous reports. For example, Xbp1(s), but not ATF6, have been reported to induce phosphatidylcholine production (Sriburi *et al*. 2004). Surprisingly, for the down-regulated genes, the signalling of Atf4, Atf6 and Xbp1 was much more similar (*figure 17 B+C*). It has been demonstrated for Xbp1, that the primary effect of Xbp1-binding to a gene promoter is generally up-regulation of the respective gene (Acosta-Alvear *et al*. 2007). If down-regulation only occurs as an effect further downstream, it might be well attributed to the action of miRNAs. The conducted miRNA-arrays still require confirmation experiments, so conclusions about individual miRNAs have to be considered as preliminary. The majority of the potential changes in miRNA – expression is weak (and not significant in these experiments) and therefore likely of minor practical relevance if looked upon as individual events. The best two candidates are miRNA 130b and 148a, both significantly, but rather moderately, up-regulated by ATF6 and XBP1 (but not by ATF4). MiRNA148a is suppressed in various types of cancer (Zheng *et al*. 2011; Zhou *et al*. 2012) and mostly associated with inhibition of cell cycle (Liffers *et al*. 2011; Guo *et al*. 2011) and promotes apoptosis by targeting Bcl-2 (Zhang *et al*. 2011). However, Bcl2 was not found to be down-regulated in the microarray experiments in response to ATF6 or XBP1 over-expression. MiRNA130b decreases glucocorticoid receptor expression and causes resistance to glucocorticoid induced apoptosis (Tessel *et al*. 2011). It

also moderately down-regulates the negative growth regulator CCN3 (This study: Nov: TG 22h: -0.31; TG 6h: -0.34) (Suresh *et al*. 2011) and tumor suppressor RUNX3 (This study: Runx3: ATF4 22h: -0.41; ATF6 22h: -0.35) (Lai *et al*. 2010). It is associated with higher resistance to chemotherapeutic agents, enhanced tumorigenicity in vivo, and a greater potential for self renewal by reducing TP53INP1 (Yip *et al*. 2011; Ma *et al*. 2010). None of the potential targets named here was strongly regulated by ATF6 or XBP1 (S*upplementary table 7*). The finding that the general regulation of miRNAs by Atf4, Atf6 and Xbp1 is significantly (positively) correlated, may already serve as a possible explanation for the similar down-regulation of mRNAs by these factors. Despite the effect of ATF4, ATF6 and XBP1 on miRNA expression occurs to be rather weak and without big individual effects, practical significance of the subtle regulation of many miRNAs can therefore not be excluded.

The results of the exon-arrays (for which confirmations with independent methods are still required) indicate that the effects on mRNA might go far beyond different expression levels but could also include a broad spectrum of alternative splicing events. The potential effects of alternative mRNA-splicing are very complex and diverse. Alternative splicing can completely change the function of the affected protein. An example is the generation of transcription factor isoforms with different activities and DNA-binding specificities (reviewed by Rio 1993). As mentioned in the results part (4.4.3), some of the top candidates for alternative splicing can be linked to important processes like ERAD (Derl3) (Oda *et al*. 2006) activation of Atf4-mediated transcription (Cep290) (Sayer *et al*. 2006a) or ER to Golgi – trafficking (Sec24a and Zw10) (Sharpe *et al*. 2011; Hirose *et al*. 2004) or regulation of transcription and mRNA-splicing. Therefore the findings about the regulation of alternative splicing have the potential for practical importance and the consequences of the found events (if they can be confirmed) should be further investigated. ATF6α mRNA is alternatively spliced during reducing stress in lymphocytes. This shorter ATF6alpha message lacks exon 7 and may have a regulatory role in the UPR (Lemin *et al*. 2007). My exonarrays did not reveal this event as a result of UPR-transcription factor over-expression.

5.3 Are ATF4, ATF6 and XBP1 inducing CHOP and subsequent apoptosis?

Maladaptive ER-stress can induce apoptosis. The question if ATF4, ATF6 or XBP1 induce CHOP-mediated apoptosis consists of two parts. Firstly: Is CHOP induced by ATF4, ATF6 and XBP1?

ATF4, p50ATF6 and XBP1(s) have been described as the transcription factors for induction of a pro-apoptotic mediator linking ER-stress to apoptosis, called CHOP, in different models. Indeed over-expression of p50ATF6 was demonstrated to cause a robust CHOP-induction in Hela-cells (Yoshida *et al*. 2000) and ATF4 over-expression was shown to have some activating effect on the Chop-promoter, especially the C/EBP-ATF-motive (Fawcett *et al*. 1999). In other cell culture models utilizing ER-stress inducing agents, an induction of ATF4, cleavage of ATF6 and splicing of Xbp1 was observed in parallel to a robust CHOP-induction (Magne *et al*. 2011). Adachi *et al.* 2008 compared the transcriptome of MEFs from ATF6+/+ and ATF6-/- mice and thereby identified ER-chaperones, ERAD-components and ER-proteins as specific ATF6-target proteins. In their system the induction of CHOP was also found to be impaired (from about 5-fold to 3 fold) in the ATF6-/- cells (Adachi *et al*. 2008). Celecoxib induces Chop in an ATF4 dependent manner (Oh *et al*. 2010).

In cells with expression of dominant negative PERK, the inducibility of ATF4 is gone, while CHOP induction is delayed. Hyperoxia (95 % oxygen) is sufficient to induce ATF4 and CHOP, including a strong induction on protein level in MLE12 cells and primary murine AECII. This effect was mediated by activation of eIF2α kinase PKR and did not include activation of the other UPR-arms (Lozon *et al*. 2011). ATF6 cleavage and Xbp1-splicing are strongly enhanced in these cells (Yamaguchi *et al*. 2008).

All these observations were leading to the general view, that the UPR-transcription factors ATF4, ATF6 and XBP1 are the main and sufficient factors for CHOP-induction (reviewed by Oyadomari, Mori 2004).

However, there are several examples from the literature questioning this view. Su *et al*. 2008 transfected HEK293T-cells with HA-tagged ATF4 and were unable to detect CHOP in nuclear extracts 36 h afterwards. They also immunoprecipitated the over-expressed ATF4, but were unable to detect co-precipitated CHOP unless they over-expressed CHOP as well (Su, Kilberg 2008). In neuronal cell models of hypoxia, Salubrinal (inhibitor of eIF2α dephosphorylation) was enhancing the induction of ATF4 as expected, but did not affect the CHOP-levels. It also reduced

induction of ATF6-cleavage (Halterman *et al*. 2008). This is another example were ATF4-levels failed to influence CHOP. In human SH-SY5Y neuroblastoma cells, simultaneous hypoxia plus salubrinal reduced CHOP induction induced by hypoglycaemia (Kögel *et al*. 2006). This suggests that the low inducibility of CHOP by hypoxia + Salubrinal mentioned above might represent a special feature of hypoxia models. Artificially sustained IRE1 signalling was reported to enhance cell survival in prolonged ER-Stress (Lin *et al*. 2007). XBP1 deletion results in apoptosis of differentiated Paneth cells *in vivo*. This included a robust induction of CHOP (about 10fold). The authors did not investigate whether this was accompanied by increased levels of ATF4, ATF6-cleavage or eIF2α phosphorylation (Kaser *et al*. 2008).

The PERK-ATF4 pathway is often regarded as the main CHOP-inducing pathway. However, studies with ß-amyloid induced ER-stress and either activation of the pathway with Salubrinal or deactivation via PERK-knockdown, suggest that the anti-apoptotic effects of the pathway are also dominating this model in human neuroblastoma cells (SK-N-SH). This was assessed by cell viability and caspase-3+4 activity tests. Again, the specific influence of ATF4 was not investigated in this context (Lee *et al*. 2010). A beneficial role of the PERK-pathway was also reported by Harding *et al.* 2000.

The mode of action of salubrinal is not completely resolved. Initially protective effects of the drug against ER-stress inducers and viral assault were reported (reviewed by Wiseman, Balch 2005). In contrast, Drexler *et al.* 2009 showed a synergistic apoptosis induction by salubrinal and proteasome inhibitors or TG. This could easily be understood as the increased ER-load, combined with an induction of the pro-apoptotic pathway initiated by eIF2α, if they had not proven at the same time that salubrinal did not increase eIF2α phosphorylation (but ATF4 and CHOP-induction) in their system (Drexler 2009).

A cytosolic isoform of GRP78 induces eIF2α phosphorylation. In this model ATF4 induction by TG was increased but CHOP induction was not altered (Ni *et al*. 2009).

The common view is, that ER-stress is leading to apoptosis, when it is too strong or prolonged (Araki *et al*. 2003; Bernales *et al*. 2006). Rutkowski *et al.* 2006 performed a series of experiments with different exposure times and concentrations of stress inducers (TG and TM). Their results showed a threshold for the intensity of ER-stress to become maladaptive. But doses of TG and TM which were sub-lethal at short term exposure were rather leading to an adaptive response, which was even protective against stronger stress at a later time point.

The mild ER-stress activated all ER-stress sensors but did not lead to apoptosis because the expression of apoptotic proteins was short lived whereas the pro-survival proteins (chaperones) were more persistent because of higher mRNA and protein stabilities (Rutkowski *et al*. 2006).

The cells (MLE12, MLE15, MEL188) utilized for this work, were reacting in the described, apoptotic manner in response to ER-stress inducing agents (mainly TG). However, in my experimental settings, the over-expression of Atf4, cleaved Atf6 and Xbp1(s) was not sufficient to induce CHOP on protein level and only had a minor effect on Chop mRNA-levels as compared to the effects of TG (see *figure 21*). The expression of the transgenes however, was comparable or higher than the inductions achieved with TG. The difference is even more striking, when we express the data as fold changes: CHOP-induction by TG was approximately 16-fold, whereas transgene-expression typically did not result in a more than 2-fold increase (calculation based on an assumed amplification efficiency of 2 in the underlying qPCRs). Consequently, the over-expression of Atf4, Atf6 and Xbp1 was not leading to increased cell death (see *figure 24*) and cell growth was not affected (see *figure 26*). Even more combined over-expression of ATF4, ATF6 and XBP1 was not changing matters, still there was almost no Chop-induction (see *figure 24*). The transgenes were rather helping the cells to overcome the transfection stress (see *figure 24 A*). As the transfection generally included the over-expression of a fluorescent reporter protein (either EGFP or DsRed2), it is generating an increased protein load which could contribute to the negative effects of the procedure. This problem could of cause be eased by the expression of ATF4, ATF6 and XBP1. Microarray analysis clearly confirmed the general functionality of the transfected transcription factors. For many targets, the extent of the regulation was comparable to the effects of TG. An insufficient dose or impaired general function of the transcription factors can therefore be excluded as an explanation for their inability to induce CHOP in my system.

The possibility that the optimal growth and survival-conditions in the cell culture medium were counteracting the pro-apoptotic action of Atf4, Atf6 and Xbp1 resulted in conductance of an experiment, where “starving medium” and medium with peroxide addition (or a combination of both) were used as a second hit. Even though these treatments were leading to a moderate increase in cell death (typically about 5 %), over-expression of the transgenes were not enhancing those effects (see *figure 25*). At this step we can therefore draw the following two conclusions.

Firstly: In spite of the results of this work, Atf4, Atf6 and Xbp1 may be able to somewhat contribute to CHOP induction and subsequent cell death but they are not sufficient to cause CHOP-induction or cell death in the utilized models. It is therefore likely that important other factors, which are not generated by the UPR-transcription factors but by pro-apoptotic ER-Stress inducers such as TG, exist.
Secondly: the postulated missing factor(s) do not just occur as a result of any unfortunate condition. This indicates that they are specific for an overwhelming ER-stress situation.
The notion that there might be more factors than ATF4, ATF6 and XBP1, with central roles in UPR-signalling, is not new. Shang *et al*. 2004 reported a "discordance of UPR signalling by ATF6 and Ire1p-XBP1 with levels of target transcripts". In this study, based on the treatment of fibroblasts with diverse ER-stress inducers, the authors concluded: "our data with the dermal fibroblast appear to refute the hypothesis that activation of ATF6 and / or Ire1p determines the levels of mRNAs derived from target transcripts. Rather, the results imply the existence of other ER-stress-sensitive factors that serve this function." The authors proposed ATF4 as the factor to fill the gap but also mentioned stress dependent interactions of ATF6 with YY1 and NF-Y (Shang, Lehrman 2004). Potential alternative mechanisms of CHOP induction are discussed in a separate chapter (5.4).
Furthermore a knockdown of Atf4 and Atf6 (at least on mRNA-level) was not sufficient to reduce the Chop-induction by subsequent treatment with TG or BFA (see *figure 23*). Assuming that the knockdown also reduced the ATF4 / ATF6-protein level this would implicate that an increase in ATF4, ATF6 and Xbp1 levels is not even necessary for Chop induction in these cells. Another argument for a rather weak impact of the three UPR-transcription factors on Chop induction is the limited overlap between early TG effects and Atf4, Atf6 and Xbp1-signaling. If ER-stress signalling is mainly mediated by transcription of Atf4, Atf6 and Xbp1-targets, the effects of these transcription factors should correlate nicely with the effects of an ER-stress inducing agent like TG. This should especially be true for the very early effects of TG, where ongoing cell death does not yet play a role. However, a moderate positive correlation of the early TG effects and the downstream signalling of the three UPR-transcription factors was exclusively detected for Atf4 and only when the analysis was limited to up-regulated genes (highlighted by *figure 27*).

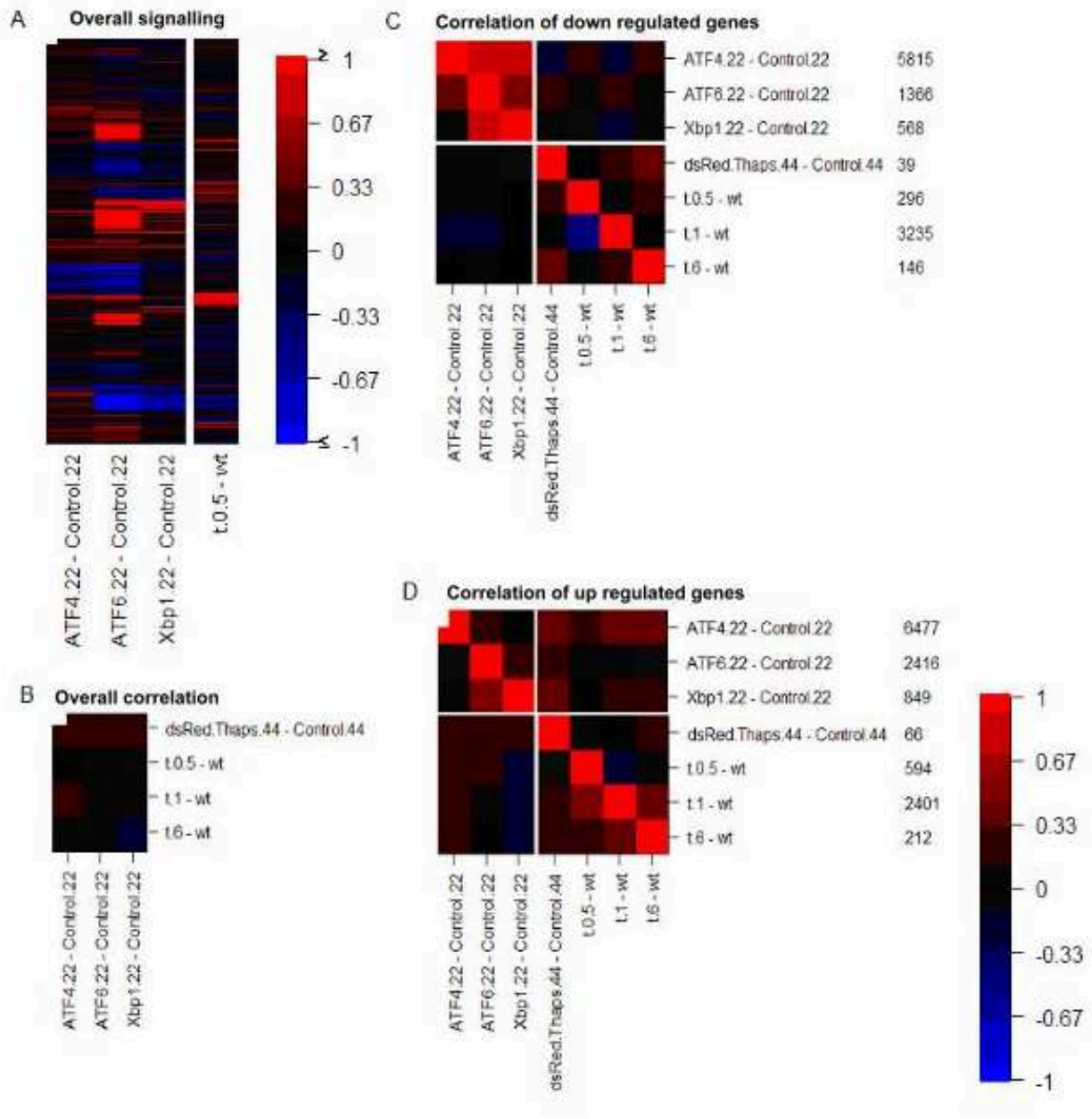


***Figure 27*: Correlation between very early Thapsigargin effect and effects of UPR-transcription factors.** A) Heat map: Overall signalling as a result of UPR-transcription factor transfections and the very early (0.5 h) effects of TG. B) Correlation chart: Overall correlation between the three transcription factors and different time points after TG-challenge. C) Correlation chart including genes down-regulated in the first compared contrast (and the number of included genes on the right side). D) Correlation chart including genes up-regulated in the first compared contrast (and the number of included genes on the right side). The scale bar on the lower left is valid for B, C and D.

Treatment with TG also activates the pathways of the ER-overload response (reviewed by Knorre 2001), which are rather not related to ATF4, ATF6, XBP1 signalling. This could partly explain differences between TG treatment and transgene over-expression as assessed by correlation analysis. A potential effect on CHOP-induction is discussed in chapter 5.4. Furthermore the expression of Atf4, Atf6 and XBP1(s) mRNA after TG or BFA challenge did not precede the induction of Chop but started rather simultaneous one (TG) or two (BFA) h after challenge. On protein level, treatment of MLE12 cells with TG for 3 h was not sufficient to induce detectable levels of ATF6 cleavage or ATF4 protein and BFA only induced ATF4 even though the siRNA-experiments described above indicated an enhanced Chop-mRNA expression 2 h after the same treatment. Of cause this observation is not sufficient to exclude an induction of Chop by already existing ATF4, ATF6 or Xbp1. It can also not be absolutely excluded that minor changes in the expression of these factors, below detection limit, contribute. But at least it indicates that other, earlier occurring factors are likely to be involved. The earliest signalling after TG treatment (after only 0.5 h) was neither dominated by Chop, nor by Atf4, Atf6 or Xbp1 up-regulation (see *figure 27*). However, none of the very early regulated genes in the microarrays serves as easy explanation for Chop-induction either (at least at quick glance).

Finally the concept that the very same three transcription factors should mediate both, the pro-survival as well as the pro-apoptotic ER-stress responses, always suffered a lack of inherent logic. Schröder *et al.* 2005 called this problem “an unsolved and unaddressed mystery” (Schröder, Kaufman 2005). A cell can either survive or commit apoptosis, not both. There are also examples for constitutively active UPR-signalling (Teodoro *et al*. 2011; Acosta-Alvear *et al*. 2007). If no simple dependency like concentration or combination is involved, the same three factors can never be responsible for the decision between both contrary directions (which is not excluding a role in the execution of both).

As described, the transgenes were over-expressed in high doses and all combinations and did never increase cell death in my hands, which makes such simple mechanisms very unlikely. Cellular models, where the effect of these factors on the pro-apoptotic downstream pathways is not of main importance could be useful to elucidate what generally triggers the decision between death and survival in ER-stress. Regardless of the general consequences for our view on ER-stress, these observations have consequences for the model of IPF pathogenesis of sporadic (and some familial forms of) IPF (as further discussed in chapter 5.6).

The *in vitro* experiments for this thesis were mainly performed in the MLE12 cell-line and only partly (lack of CHOP induction and no increased cell death after Atf4, Atf6 and Xbp1 transfection) confirmed with MLE15 and MEL188 cells. This raises the question in how far the findings discussed in this chapter are applicable to a general model of ER-stress signalling. The relevance of the findings might partly be limited to AECII derived cell lines (MLE12 and MLE15) or AECII. It is also possible, that the observed features are more general specialties for secretory cells (as the results for MEL188 would suggest).

Taken together we can state, that ATF4, ATF6 and XBP1 seem to play only a minor role in the induction of CHOP in my system. This leads us to the second part of this chapter: Does CHOP induce apoptosis?
CHOP was mainly described as an obligate mediator of apoptosis but it is not necessarily always inducing cell death and it can play much broader roles. CHOP and ATF4 can interact and cooperate in decreasing activation by the CRE-motive and the HTLV-I proximal Tax-responsive element (Gachon *et al*. 2001). The function of CHOP is not limited to cell death decisions. For example, CHOP is crucial for Il23 transcription in dendritic cells (Goodall *et al*. 2010) and, in Podocytes, CHOP reduces expression of ß1 and α3-integrin and enhances cell adhesion to collagen IV-coated plates (Morse *et al*. 2010). CHOP is also involved in the regulation of iron homeostasis (Oliveira *et al*. 2009).
In a model of hypoxia induced cell death CHOP even played a protective role. This was also true for TG-treatment in this set of experiments. The authors suggested that posttranslational modifications of CHOP are responsible for the switch between an adaptive and a pro-apoptotic response to CHOP (Halterman *et al*. 2010). Mice lacking CHOP had increased lung permeability (not associated with differences in neutrophil recruitment) and decreased survival in response to hyperoxia (Lozon, Altemeier 2009; Lozon *et al*. 2011). The authors also concluded that CHOP can play a cytoprotective role in this model but did not investigate the fate of individual cell types *in vitro or in vivo* to confirm this view.
The apoptotic effect of CHOP can also be regulated downstream as demonstrated in macrophages over-expressing HSP70 as a protective factor. HSP70 reduced apoptosis by inhibiting the migration of Bax into mitochondria in this system (Gotoh *et al*. 2004). HSP70 (gene name: Stch) is up-regulated by ATF6, XBP1 and TG (This study: Stch: ATF6 22h: +0.55; XBP1 22h: +1.16; TG 22h: +0.69).
CHOP is also not the only mediator of ER-stress induced apoptosis. Benzodiazepinones were shown to specifically inhibit ER-stress mediated

apoptosis but not apoptosis by inducers of extrinsic (tumor necrosis factor) or intrinsic (mitochondrial) cell death pathways. The reagents were reducing ASK1 levels but not CHOP-expression. ASK1 (gene name Map3k5) knockdown by siRNA was also reducing apoptosis, indicating ASK1s important role in ER-stress mediated apoptosis (Kim *et al*. 2009). The factor is only moderately down-regulated by ATF6 and TG (This study: Map3k5: ATF6 22h: -0.35; TG 22h: -0.25; TG 1h -0.36; TG 6h: -0.52) indicating a minor role in my system.
In models of force induced apoptosis, eIF2α phosphorylation and activation of CHOP-expression, but not, activation of the XBP1 and ATF6-pathways, have been observed. However, apoptosis (as shown by caspase-3 cleavage) was relying on PERK, but neither on eIF2α phosphorylation or CHOP induction in this model. The authors consequently proposed the existence of an alternative, PERK-, but not CHOP-dependent apoptosis pathway (Mak *et al*. 2008). Neither the mechanism nor its role in ER-stress induced apoptosis is known yet.
On the contrary CHOP has been shown to clearly play a pro-apoptotic role in many models. CHOP induces apoptosis in rat embryonic heart-derived H9c2 cells (Han *et al*. 2005) and over-expression of CHOP in mouse osteoblasts *in vivo* was leading to increased osteoblast apoptosis and osteopenia (Pereira *et al*. 2007).

In experiments performed under my supervision in the context of a practical training, CHOP was successfully induced in MLE12 cells by co-transfection with my Chop-pBI-L-plasmid and a transactivator plasmid (pTet-On Advanced, Clontech) and subsequent induction with Dox. This was leading to a 6 % increase in cell death assessed by LDH-assay, confirming the described role of CHOP as a mediator of ER-stress induced cell death also for the utilized cell line (Serdar Uzunzas, personal communication).
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5.4 Alternative mechanisms of CHOP induction

Chop is mainly regulated on transcriptional level (reviewed by Oyadomari, Mori 2004). The transcription factors and binding sites for CHOP activation differ from cell type to cell type and depend on the inducing stress. CHOP is also inducible by amino acid starvation or arsenite treatment, manipulations that lead to eIF2α phosphorylation without causing ER-stress or activating PERK (reviewed by Harding *et al*. 2002).
In PC-3 and MCF-7 cells, Brefeldin A is inducing Chop via the AARE, ERSE, and AP-1 elements (Kwok SC, Daskal I 2008). In a model of mitochondrial UPR

induced by over-expression of a mutant mitochondrial protein (a different reaction than the ER-UPR), CHOP was induced via the AP-1 element, in contrast to Tunicamycin, which required ERSE (Horibe, Hoogenraad 2007). 15-deoxy-Delta (12, 14)-prostaglandin J is inducing CHOP and apoptosis in HELA-cells depending on the C/EBP-site and the ERSE sequence of the Chop promoter (Saito *et al*. 2003). Consequently, ER-stress responses to the same external stimulus can be very different from tissue to tissue. For example, amphetamine increases Atf4 and Hspa5 / Grp78 mRNA in striatum but, unlike in parietal cortex and meninges and associated vasculature, not Pdia4 and Nfkb11. Chop is only increased in striatum and parietal cortex (Thomas *et al*. 2010).

If the three UPR-transcription factors are not sufficient to induce a robust pro-apoptotic signalling, that does not mean that they are not necessary. As bZIP-transcription-factors they can heterodimerize with many other factors of the same type (summarized by Schröder, Kaufman 2005). Their transcription factor activity on the Chop promoter might depend on the sufficient expression of one of those factors. A possible candidate is NF-Y. NF-Y binds to the inducible GCACG domain of the CHOP promoter and activates CHOP gene expression in response to cellular stress (Ubeda, Habener 2000).
Yoshida *et al.* 2000 reported that ATF6-binding to the consensus sequence GCACG (part of the human CHOP-ERSE-element) requires binding of NF-Y to CCAAT 9bp upstream and induces CHOP (Yoshida *et al*. 2000). The formation of a transcription factor complex including ATF6 and NF-Y at their respective ERSE is also inducing chaperone production (Yoshida *et al*. 2000). Insufficient levels of NF-Y would therefore be a possible explanation for a limited effect of ATF6 on CHOP expression. But neither TG treatment nor over-expression of ATF4, ATF6 and XBP1 significantly regulated the NF-Y transcript levels in my qPCRs. In my microarrays, NF-Y subunits were only mildly regulated by TG of UPR-transcription factors (This study: nfya: TG 22 h: +0.41; nfyb: TG 6 h: +0.44; TG; nfyc: ATF6 22 h: -0.57 TG 6 h: +0.58).
Yamamoto *et al.* 2004 additionally showed an NF-Y independent binding of XBP1 to ERSE and a reduced transcription from ERSE II in absence of XBP1 (Yamamoto *et al*. 2004). The ERSE motive is also bound by YY1 (Roy, Lee 1999). The regulation of this factor (This study: Yy1: ATF6 22h +0.59; TG 22 -0.38; TG 1h: -0.75) is moderate, which does not suppose a big impact on differential Chop expression.

Single copies of ERSE are not sufficient for stress inducibility. Only tandem copies are fully functional (Roy, Lee 1999). There is only one copy of ERSE in the proximal promoter of murine CHOP. This indicates that other promoter elements also play an important role for the induction.

ATF3 was described as a transcription factor downstream of ATF4. It was indicated to be involved in Chop induction by leucine starvation, but not TG treatment (where it induced Gadd34) (Jiang *et al*. 2004). ATF3 was down-regulated by ATF6 and Xbp1(s) (This study: Atf3: ATF6 22h: -0.98; ATF6 44 h: -0.58; XBP1 22 h: -0.65) and up-regulated by TG (This study: Atf3: TG 22 h: +1.29; TG 0.5 h: tendency for up-regulation: +0.67; TG 1 h: +1.29; TG 6 h: +1.97), however the corresponding qPCRs were not significant. Atf3 was only slightly and not significantly up-regulated by ATF4 in my system (qPCR: *figure 18*). As Atf3 is regulated very early after TG-treatment it has the potential to be partly responsible for the decision for or against Chop induction in my system. ATF4 was shown to bind the C/EBP-ATF-site of the CHOP promoter after arsenite treatment of PC12 cells, and acts as a transcriptional activator there. In the recovery phase, ATF3 binds this element and represses CHOP transcription including ATF4 mediated transactivation (Fawcett *et al*. 1999). ATF3 was robustly up-regulated by TG-treatment after 1, 6 and 22h and moderately down-regulated by ATF6 and XBP1 (22h). This would rather suggest a lower CHOP induction by TG (see s*upplementary table 7*).

Another candidate is ATF5. Yamazaki *et al*. 2010 showed that ATF5 activates the CHOP gene promoter in HepG2 cells and revealed that the amino acid response element (AARE)-1 is responsible for ATF5-dependent promoter activation (Yamazaki T *et al*. 2010). ATF5 was robustly up-regulated by TG after 6 and 22h (see supplementary table 7) in my microarrays, but not earlier. This indicates its potential impact on later CHOP induction but does not provide any explanation for the very early CHOP-induction by TG. Another possible candidate is ATF2. It is essential for the amino acid responsiveness of the CHOP promoter (Bruhat *et al*. 2000). It is not the *de novo* expression of ATF2, but its phosphorylation, which is leading to CHOP activation (Averous *et al*. 2004). Therefore, the fact that ATF2 is not strongly regulated in my microarrays does not exclude that it might play a role anyways. Nfκb subunit p65 can repress CHOP-promoter activity (This study: Rela: ATF6 22 h: -0.91; Xbp1 22 h: -0.81; TG 6 h: +1.25) and over-expression of Iκb (This study: Nfkbie: ATF6 22 h: -0.5; TG 22 h: -0.34; TG 6 h: +1.79 / Nfkbia: TG 22 h: -0.91; TG 1 h: +1.04; TG 6 h: +1.23) induces CHOP (Nozaki S *et al*. 2001). Except for the transient up-regulation of Nfkbie and Nfkbia by TG, the results

about NFκB-signalling would rather imply an increased CHOP induction by ATF6 and Xbp1 and a decreased one by TG. Therefore it is unlikely that the ER-overload response is responsible for the relative inability of the UPR-transcription factors to induce CHOP in my system.

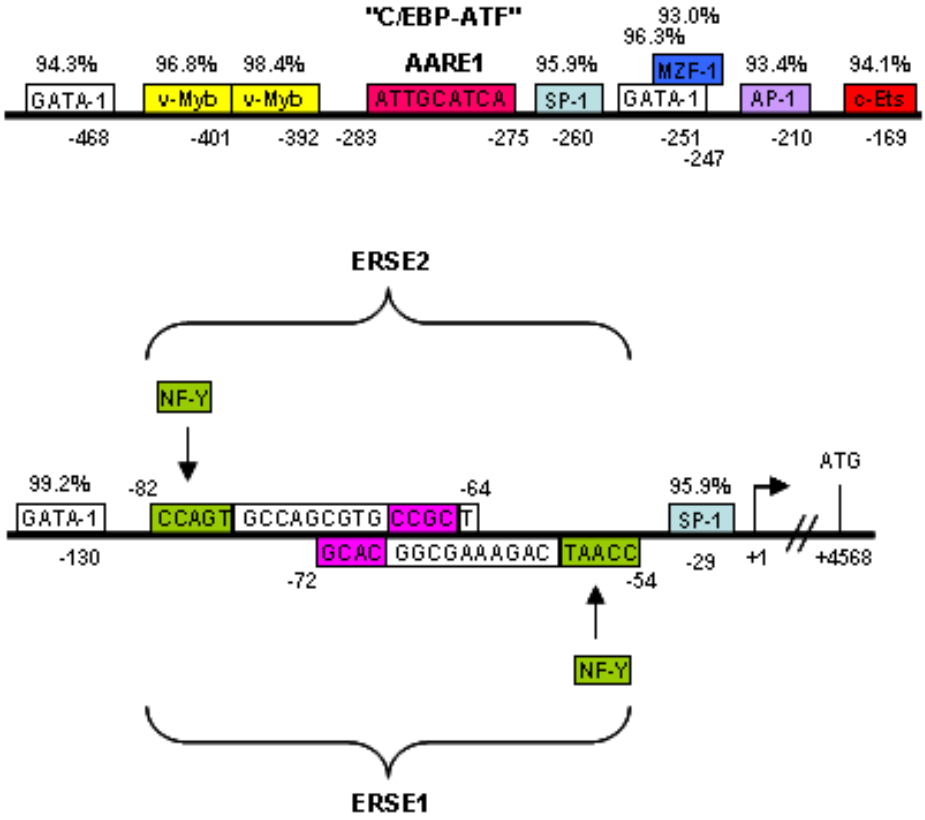


***Figure 28*: *In silico* analysis of the murine CHOP- promoter.** The figure is a representation of the proximal murine CHOP- promoter. Boxes depict the identified consensus binding sites. The percentages above stand for the degree of identity between the found and the consensus sequence. The arrow shows the transcription start and ATG the start-codon for translation. The numbers below the line stand for the position relative to the transcription start (in bp). (Martina Korfei, unpublished)

An *in silico* analysis of the proximal murine Chop-promoter, conducted by Martina Korfei of our laboratory (unpublished data, personal communication), was revealing potential additional transcription factor binding sites not related to Atf4, Atf6 and Xbp1 binding (*figure 28*), namely three copies of GATA1, 2 copies of v-myb, 2 copies of SP-1 and one copy of MZF and c-ets sites.

The independent binding of transcription factors to such sites might enhance the effects of the three factors ATF4, ATF6 and XBP1. However, no known candidates for binding to such sequences were specifically and strongly regulated in comparison of TG and transgene effects.

It is possible that not the *de novo* expression of a certain transcription factor is the trigger for Chop-induction, but other mechanisms like posttranslational modifications of existing factors or the presence of a necessary cofactor.

Ca^{2+} release from the ER is an important step in the mechanism of the execution of apoptosis in more than one way. Cytoplasmic Ca^{2+} can also get into the nucleus via the nuclear pore and modulate gene transcription there (reviewed by Groenendyk, Michalak 2005). ER-stress induction by TG directly induces a Ca^{2+} release from the ER, where it is required for protein folding. It also directly acts as a death stimulus. It is well possible, that it also directly influences the transcription of Chop, which would be consistent with the very early Chop induction after TG-treatment. In U251MG cells, a combination of amiodarone and TRAIL was leading to an apoptotic response, including the up-regulation of ATF4 and CHOP. In this setting amiodarone was strongly increasing intracellular Ca^{2+} levels and the sensitivity to apoptosis was highly dependent on the presence of extracellular Ca^{2+} (Kim IY *et al*. 2011). Influx of extracellular Ca^{2+} is not inhibiting ER-function like TG-treatment. Therefore, a direct transcriptional effect on ATF4 and eventually CHOP-levels appears likely. However, in the BFA-system, which was also used here, Ca^{2+} release from the ER should only occur as a result of an ongoing pro-apoptotic signalling and cannot dominate its beginning.
Independently of the exact impact on the CHOP promoter, several other factors were reported to influence CHOP expression. None of them could be convincingly made responsible for the lack of CHOP induction by ATF4, ATF6 and XBP1 as compared to TG in my system. Zhu *et al*. 2000 presented a model in which the activation of p38 MAPK was leading to a transcriptional activation of CHOP (Zhu, Lobie 2000). They did not investigate if this was mediated by eIF2α phosphorylation or ATF4. The weak regulation of this factor (This study: Mapk14: ATF6 22 h: -0.24; TG 22 h: -0.3; TG 6 h: +0.44) does not support a role for it in my system, but the activation could, of cause, well happen without transcriptional changes. Recent evidence shows that eIF2α-phosphorylation can directly or indirectly activate CHOP translation by enabling a ribosomal bypass of the upstream open reading frame (ORF) in the CHOP promoter (Palam LR *et al*.

2011). If eIF2α phosphorylation could influence CHOP translation independently of ATF4, this would partly explain the low CHOP induction in my ATF4, ATF6 and XBP1 over-expression models. However, it does not explain the observed differences on transcript level. ERdj5 (This study: Dnajc10: ATF6 22 h: +0.37; ATF6 44h: +0.52; TG 6h: -0.55) inhibits TG induced eIF2α phosphorylation and the subsequent inhibition of protein synthesis and expression of BIP in SH-SY5Y cells and leads to apoptosis. It paradoxically also inhibits ATF4 and CHOP expression (Thomas, Spyrou 2009). Again, the weak regulation of this factor does not make its impact in my system very likely. PCAF was reported to interact with the C-terminal transactivation domain of XBP1(s) and enhance its transcription activity, including CHOP transcription (Lew QJ *et al*. 2011). However, microarray probes for Pcaf were not regulated by the UPR-transcription factor over-expression and slightly (This study: TG 22 h: -0.40; TG 6 h: -0.62) down-regulated by TG-treatment, excluding the possibility that different PCAF levels are responsible for the lack of ATF4, ATF6 and XBP1 mediated CHOP induction in my system. XBP1s is a target of acetylation and deacetylation mediated by p300 and SIRT1 respectively. P300 increases acetylation and protein stability of XBP1s, and enhances the transcriptional activity of XBP1s. SIRT1 deacetylates XBP1s and inhibits the transcriptional activity of XBP1s (Wang *et al*. 2010). Sirt1 was supposed to be a negative regulator of UPR-signalling, affecting Bip as well as Chop (Li *et al*. 2011). A reduced XBP1 transcriptional activity and stability has the potential to play a role in differential Chop induction. However, both factors were slightly down-regulated by ATF6 and XBP1 in my microarrays (-0.55 to -0.75). P300 was also down-regulated by ATF4 (This study: ATF4 22h: -0.73) and Sirt1 was slightly up-regulated by TG treatment (This study: Sirt1: TG 22 h: +0.62). Hence, the data does not support a dominant role of Sirt1 / p300 ratio for the different Chop induction by UPR-transcription factors and TG. ATF4 degradation depends on its phosphorylation and subsequent binding by ßTrCP (genename: Fbxw11; This study: not regulated) ubiquitin ligase (Lassot *et al*. 2001). Increased degradation of ATF4 could reduce its ability to induce Chop. The protein FIAT can interact with ATF4 and inhibit its transcriptional activity on the OSE1-site of the osteocalcin promoter (Yu *et al*. 2005). Effects of FIAT-binding on ER-stress specific promoter elements has not been investigated but cannot be excluded. Increased expression of FIAT could possibly also reduce the ATF4 dependent activation of CHOP. However, FIAT (gene name: Txlnb) is not regulated in my system. Activation of TLR4-signaling by low dose lipopolysaccharide can attenuate the translation of ATF4 and reduces CHOP

levels without inhibiting eIF2α phosphorylation (Woo *et al*. 2009). The authors did not investigate whether the lower induction of CHOP was dependent on ATF4 expression. The regulation of TLR4 signalling was not investigated in this work. In pancreatic ß-cells Il1 is inducing CHOP. This effect was mainly seen in protein level (Shao C *et al*. 2010). My microarray results do not provide hints for Il1 involvement (see *supplementary table 7*) in my system however Il1-protein levels were not observed. Ppp1r15b plays an important role in eIF2α dephosphorylation (Harding *et al*. 2009). Decreased expression of this factor might therefore influence apoptotic signalling including Chop expression. Only minor down-regulation of this factor happened as a result of ATF6 over-expression and TG treatment (This study: Il1: ATF6 22 h: -0.35; TG 22 h: -0.42; TG 6h: -0.30). Chen *et al*. 2010 showed that Chop translation can also be triggered by eIF4e-phosphorylation. But this mechanism is not involved in TG-mediated CHOP-induction (Chen *et al*. 2010). Therefore, it is unlikely that it is responsible for the different induction of CHOP by ATF4, ATF6, XBP1 and TG, which I observed. Chop has been demonstrated to be sensitive to nonsense mediated decay (NMD). Upf1 knockdown is increasing the stability of Chop-mRNA by unknown mechanisms (Gardner 2008). The level of Upf1 was slightly decreased after Atf6 treatment (22h) and in TG treated cells 22h after TG-addition but was slightly up-regulated 6 h after treatment in the microarrays. Taken together, these results are not sufficient to postulate an effect of NMD in my system. However, this can also not be excluded. Depletion of D4S234E (a novel, p53 regulated gene, murine homolog not known) diminished genotoxic stress induced reduction of Bcl-2 and augmentation of CHOP (Kudoh *et al*. 2010). In my experiments the p53 signalling pathway was influenced by TG and by ATF4, ATF6 and XBP1. An influence of this pathway on differential CHOP induction seems possible. However, the gene regulation of the p53 pathway by TG-treatment and ATF4, ATF6 and XBP1 are positively correlated (data not shown).

Lack of WFS1 can lead to apoptosis. WFS1 negatively regulates ATF6 levels. Therefore it was supposed to play a role in recovery after ER-stress. It also negatively influences Bip, Xbp1 and Chop mRNA-levels (Fonseca *et al*. 2010). Wfs1 was robustly up-regulated by ATF6 and XBP1(s) (This study: Wfs1: ATF6 22 h: +1.68; XBP1 22 h: +1.31) over-expression in my system. In principle it could be involved in the low Chop induction by these factors, but TG-treatment also induced Wfs1 (This study: Wfs1: TG 22 h: +1.41). Scheuner *et al.* 2006 reported that PKR (This study: Eif2ak2: ATF6 22 h: -0.66; ATF6 66 h: -0.65; TG 22 h: -1.02) dependent phosphorylation of eIF2α is activating apoptosis and that a phospho-

mimetic Ser51 to Asp mutant eIF2α also induces apoptosis. They pointed out that PERK mediated eIF2α phosphorylation at the same site was protecting the cells from ER-stress (Scheuner *et al*. 2006). The lower expression of PKR especially by TG does not support a pro-apoptotic role for this factor in my system. Backer *et al.* 2009 demonstrated that cleavage of BIP via toxin (subunit A of the bacterial AB_5-toxin) induced cell death via apoptosis, synergistically with TG (Backer *et al*. 2009) and over-expression of mutant BIP causes a reduced surfactant protein synthesis and CHOP-induction *in vivo* (Mimura *et al*. 2007). It is possible that this is attributed to activation of the ER-stress sensors by depletion of BIP. Alternatively, the loss of BIP might just increase the load of unfolded proteins because its chaperone function is gone. But it also proposes the possibility that BIP directly acts as an anti-apoptotic agent. In A549 cells (a human AECII-tumor cell line), a robust knockdown of BIP was not sufficient to induce UPR-signalling (Gewandter *et al*. 2009). In my system the induction of BIP by TG was stronger (about 4 fold) than by any of the transfected transgenes.

5.5 *In vivo* studies

Transgenic animal lines for conditional over-expression of the ER-stress transcription factors ATF4, p50ATF6 and XBP1(s) have been successfully generated. Furthermore, a construct for a CHOP-over-expressing mouse line has been cloned. This line should become useful to directly induce an ER-Stress signalling mediated, apoptotic stimulus, when the other transgenic lines fail to do so. First tests for functionality indicate that at least the tested ATF6 line and the Xbp1 line really have inducible over-expression of these transcription factors, whereas the ATF4 line failed to show enhanced levels of the protein. IHC analysis indicates that the over-expression of ATF6 and XBP1 is not absolutely specific for AECII, but also occurs in a population of bronchial epithelial cells (see *figure 11 D+H*). This is not entirely unexpected as the specificity to AECII of the human SP-C promoter-rtTA was not proven by the authors which generated the line (Tichelaar *et al*. 2000). This fact has to be considered if a lung-phenotype is observed in a later experiment (e.g. with second, pro-fibrotic or ER-stress inducing hit, like BLEO or Tunicamycin treatment). Transgene effects on the bronchial epithelial cell population might then also contribute to the phenotype. The levels and specificity of transgene induction depend on the integration site in the genome. Additional lines for XBP1(s) and ATF4 are in the breeding program, but were not yet tested for functionality. Due to different transgene integration sites,

the phenotypes of separate lines with expression of the same transgene vary substantially. Considering the results from different lines with the same transgenes therefore generally helps to get a more secure view on the possible effects of an individual transgene. It also provides another chance to get a functional line for the ATF4 construct and an XBP1(s) -line, with different transgene expression levels, for further studies. Another possibility to characterize the transgene effects are experiments with longer transgene induction in ++/++ animals (up to now only completed for ATF4-line), where the transgene-expression is potentially stronger and can be sustained over a longer period of time. The breeding program also includes the (partly completed) breeding of transgenic ++ lines without a transactivator. After breeding in a different transactivator, the mouse lines can also be utilized to study the effects in other tissues than the lung. The approach is only limited by the availability of an organ (or cell type) specific transactivator line. This possibility extends the usefulness of the mouse lines to other fields were the study of the UPR is of interest (e.g. in the field of neurodegenerative diseases).

The phenotype of the mice and the lungs in response to ATF4, ATF6 and XBP1 induction in AECII has been broadly observed. The observations included the development of body weight, cell countings and cellular composition in the BAL, number of animal deaths during the experiment, lung compliance and running time in treadmill-test (see *supplementary tables 2-6*). The histologic appearance of the lungs was unaffected. Therefore the application of a fibrotic Ashcroft-score (Ashcroft *et al*. 1988) was unnecessary. On the basis of the assessed parameters, there was no change in the phenotype of any of the transgenic mouse lines as a result of transgene induction, neither after short term (4 weeks) nor after long term (up to 32 weeks) induction. This finding has to be interpreted with caution. On the one hand it has not been shown that the transgene induction is sustained over such a long period. Even though the results of the *in vivo* imaging suggest a longer expression, ATF6 protein levels in Western-Blot were only induced after 4 weeks and Xbp1 expression in Xbp1-mice on IHC-level was only investigated after 4 weeks of induction. On the other hand long term over-expression experiments with the ++/++ lines, which potentially have stronger transgene expression, have not been finalized for ATF6 and XBP1 lines. The molecular phenotype was not observed in much detail, yet. The induction of BIP in ATF6-mouse AECII (observed in IHC) is an argument for the functionality of this transgene. As mentioned in the results part (4.2.1), no increased levels of cleaved caspase-3 or CHOP could be detected after transgene induction in ATF6 or XBP1 animals so far. The lack of a molecular phenotype would of cause also explain the absence of

a physiologic phenotype. If the AECII are largely unaffected by the over-expression of the transgenes and more specifically do not commit apoptosis, there is no reason why the lung morphology or the general fitness of the mice should change. Taken together this would fit to the *in vitro* results were ATF4, ATF6 and XBP1 were unable to generate a robust pro-apoptotic effect.

5.6 Implications on the pathogenesis of IPF

The involvement of ER-stress and AECII apoptosis in IPF has been demonstrated by other authors. However, expression of the central ER-stress induced transcription factors ATF4, ATF6 and XBP1 could not be demonstrated to be involved in the apoptotic events, which potentially trigger the disease.
The chain of events according to the epithelial damage hypothesis is the following: ER-stress in the AECII leads to induction of ATF4, ATF6 and XBP1. These factors induce Chop and Chop leads to apoptosis of the AECII-cells. The AECI which are derived from AECII are also depleted and a continuous alveolar damage and general lack of a normal epithelium is the result. The role of newly discovered lung stem cells (Kajstura *et al*. 2011) in this process still has to be determined. This leads to a fibrotic remodelling of the lung. The results discussed in this chapter do not question (or support) the complete model but indicate that alternative pathways from ER-stress to CHOP induction and subsequent apoptosis must be identified. This conclusion is valid, as long as it cannot be shown that primary AECII in the context of an intact lung do not behave completely different in this regard.

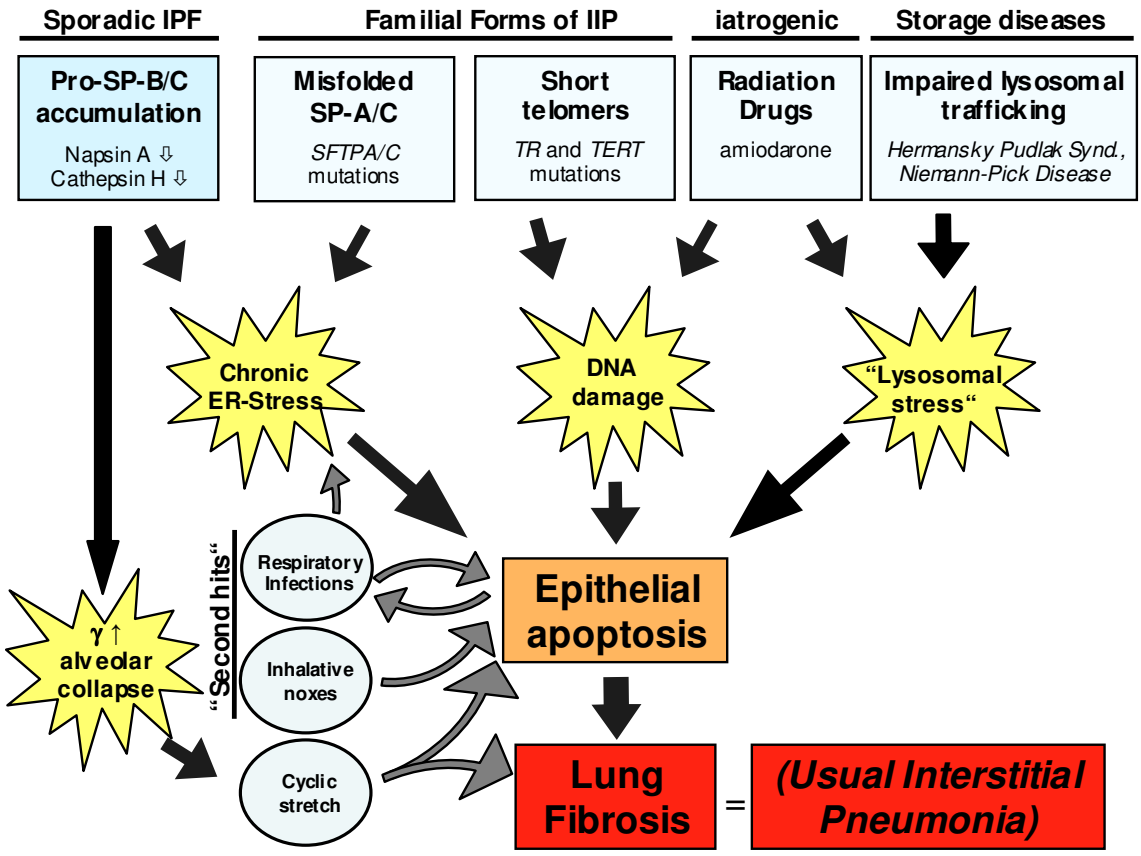


***Figure 29*: The epithelial damage hypothesis of lung fibrosis.** According to the epithelial damage hypothesis alveolar epithelial damage via apoptosis is a common feature of different deseases which manifest in lung fibrosis. The apoptosis can be caused by Lysosomal stress, DNA damage or chronic ER-stress. Some secondary events like respiratory infections, inhalative noxes and cyclic stretch caused by alveolar collapse can aggreviate the epithelial apoptosis or directly increase the fibrotic remodelling (Andreas Günther, personal communication). IPF= idiopathic pulmonary fibrosis; IIP=idiopathic interstitial pneumonia.

However, apoptosis is by far not the only result of ER-stress with potential connections to IPF-pathogenesis and ER-stress is not the only mechanism which is potentially involved in AECII apoptosis. There is also data supporting the hypothesis that alveolar epithelial cell death is induced by abnormal lung fibroblasts *in vivo* as it is *in vitro* (Uhal *et al*. 1998b). This mechanism is not necessarily independent of the ER-stress in the AECII. AECs are the primary source for transforming growth factor-ß (TGF-β), a critical cytokine in the transdifferentiation of fibroblasts into the activated myofibroblast phenotype (reviewed by White *et al*. 2003). (This study: Tgfb1: ATF6 22 h: -0.74; TG 22 h: -0.86 / Tgfb2: TG 6 h: -0.98 / Tgfb3: TG 22 h -1.64, TG 6 h -1.08). AECs produce

platelet-derived growth factor (PDGF), a potent mitogen and chemo-attractant for fibroblasts (reviewed by White *et al*. 2003) (This study: Pdgfa: ATF6 22 h: -0.34; XBP1 66 h: +0.54; TG 22 h: -1.03 / Pdgfb: ATF6 22 h: -0.49; TG 22 h: -0.83).
The strong pro-apoptotic ER-stress induced by TG rather seems to decrease these factors. TNF-α (Tnf; not regulated in my microarrays) is secreted by hyperplastic type II AECs in pulmonary fibrosis and promotes DNA synthesis and proliferation of fibroblasts (reviewed by White *et al*. 2003). Endothelin-1 has also been shown to stimulate fibroblast DNA synthesis and proliferation as well as to induce transdifferentiation of fibroblasts to myofibroblasts (reviewed by White *et al*. 2003) (This study: Edn1: ATF6 22 h: -0.88). Prostaglandin E2 is a potent inhibitor of fibroblast collagen synthesis and proliferation and reduced in IPF-patients (reviewed by White *et al*. 2003). The regulation of Prostaglandin E2 (This study: Ptges2: ATF6 22 h: -0.7; TG 22 h -0.53; TG 6h: +0.51) by ER-Stress might contribute to fibroblast collagen synthesis.
Another big chapter is the mutual influence of oxidative stress and ER-stress and the presence of both in IPF. Accumulation of unfolded protein in the ER lumen is sufficient to produce ROS and that both ROS and unfolded protein are required in concert to activate the UPR and apoptosis. Antioxidant agents (Glutathione, N-acetylcysteine, Apigenin), were reported to reduce CHOP induction and cell death by TG and BFA, suggesting an important role of ROS in ER-stress mediated apoptosis (Choi *et al*. 2010; Malhotra *et al*. 2008; Cullinan, Diehl 2006; Park *et al*. 2010). On the reverse, Chop deletion reduces oxidative stress, attenuates UPR induction, and prevents apoptosis in hepatocytes during conditions of unfolded protein accumulation in the ER lumen (Malhotra *et al*. 2008). In my hands, peroxide treatment was not sufficient to switch the pro-survival response induced by ATF4, ATF6 and XBP1 to an increased apoptotic response. The different action of TG and these factors is not attributed to generation of ROS.
Oxidative stress is indicated to participate in the epithelial damage in Idiopathic interstitial pneumonia (Kuwano *et al*. 2003) and antioxidant treatment is considered useful in IPF (Kinnula *et al*. 2005). IPF is characterized by an increased spontaneous production of oxidants by lung inflammatory cells (Cantin *et al*. 1987). This and reduced antioxidant levels in the lower respiratory tract have been thought to be involved in IPF pathogenesis. Sputum glutathione levels are 4fold reduced and correlate with disease duration and lung function impairment (Beeh *et al*. 2002). Also Glutathione levels in the IPF BAL are reduced (Montaldo *et al*. 2002). Nrf2 is a critical effector of PERK-mediated cell survival (Cullinan *et al*. 2003). Its activation contributes to the maintenance of glutathione levels, which

in turn functions as a buffer for the accumulation of ROS during the UPR (Cullinan, Diehl 2004). NRF2 has a critical role in protection against pulmonary fibrosis, presumably through enhancement of cellular antioxidant capacity (Cho *et al*. 2004). It is moderately down-regulated by combinations of ATF4, ATF6 and XBP1 transfections and TG (*figure 18G*). The level of Glutathione synthetase (gene name: Gss) is not regulated in my *in vitro* system.
Oxidative damage of BAL proteins occurs in non-smoking patients with IPF (Lenz *et al*. 1996). Increased production of NO and peroxynitrite may be responsible for the oxidative damage in IPF (Saleh *et al*. 1997). Extracellular superoxide dismutase (gene name Sod3, not regulated in my system) is very low in fibrotic areas of UIP, which may further increase the oxidant burden in this disease (Kinnula *et al*. 2006).
Other factors are Caveolin-1, Gelsolin and plasminogen activator inhibitors. Caveolin-1 was proposed as a protective regulator of pulmonary fibrosis. It is down-regulated in IPF and an effective treatment in the BLEO model (reviewed by Verma, Slutsky 2007). Caveolin-1 is not regulated by ATF4, ATF6 or XBP1 but down-regulated by TG (This study: Cav1: TG 6 h: -1.28; TG 22 h: -0.66). This indicates a down-regulation by maladaptive ER-stress. Gelsolin expression is increased in the IPF alveolar epithelium and necessary for the development of modelled pulmonary inflammation and fibrosis, while the caspase-3-mediated gelsolin fragmentation was shown to be an apoptotic effector mechanism in disease pathogenesis (Oikonomou *et al*. 2009). Paradoxically, the expression of this apoptotic factor is down-regulated in my model (This study: Gsn: TG 22 h: -1.27; TG 6 h: -1.26). Lack of plasminogen activator inhibitors (allowing greater plasmin activity) prevents the formation of significant fibrosis. It is known that AECs synthesize both PAI-1 (This study: Serpine1: ATF6 44 h: -0.59; TG 22 h: -1.22; TG 1 h +0.65; TG 6 h: -1.13) and urokinase. (reviewed by White *et al*. 2003). (This study: Plasminogen activators: Plat: ATF6 22 h: -0.34; TG 22 h: -1.40; TG 6 h: -1.42 / Plau: TG 22 h: -0.44).

5.7 Outlook

Over-expression of ATF4, p50ATF6 or spliced XBP1 is obviously not followed by robust CHOP induction or initiation of cell death in the models employed in this thesis. Instead, the resulting downstream signalling turned out to be rather in favour of cellular survival. This is consistent with the finding that there was no detectable phenotype in the *de novo* generated transgenic mouse-lines which

conditionally over-express these factors, so far. If one takes into regard results from previous publications, the currently used paradigm, according to which “overwhelming” and “persistent” activation of the three branches of the unfolded protein response (UPR) is decisive for the induction of CHOP and pro-apoptotic events in ER-stress, has to be questioned. Several alternative possibilities for switching an ER-stress related pro-survival signal into a pro-apoptotic one were considered in light of the herein presented data, but these putative explanations deserve further investigation in different cell types and categories (such as secretory and non-secretory cells). Based on the data of this work, novel concepts of ER-stress mediated apoptosis may evolve and underly a more specific approach to block the pro-apoptotic part of the ER-stress response.

With regard to IPF, the genetically engineered mice developed in the context of this work and thereafter offer the possibility to address several additional important questions. Firstly, mice with over-expression of CHOP based on the construct developed in this work, will be employed to rigorously test if pro-apoptotic ER-stress downstream signalling through CHOP alone is sufficient to induce apoptotic AECII death and to cause development of lung fibrosis (currently there is one founder mouse being cross-bred) If this was the case, the model might not only serve as proof of principle for ER-stress induced lung fibrosis, but also serve as a more reliable and closer model for IPF as compared to the currently widely used bleomycin model, which certainly shares some features with IPF, but poorly reflects the IPF pathomechanism. Also, in this case, novel treatment modalities aiming to block CHOP in AECII will certainly evolve and can be rigorously tested in this model. Secondly, the generated mouse lines over-expressing ATF4 (once a line with convincing expression levels of this factor is established), ATF6 and XBP1 can be used to check the effects of these factors in other models of lung fibrosis triggered through induction of ER-stress (like the pepstatin model). The crucial question to be answered here would be, if additional activation of UPR branches in the context of an existing ER-stress may provoke a rather beneficial (cytoprotective) or a rather detrimental (pro-apoptotic) response. This again would have direct implications for the development of future therapies, as full blown epithelial ER-stress is a hallmark in IPF.

Thirdly, the tissue and cell type-specific induction of ATF4, ATF6 and XBP1 in the transgenic animals depends on the activator line. Especially the existing single homozygous sub-lines can easily be used to extend the research about the effects of the UPR-transcription factors on organ systems other than the lung. Provided that e.g. CHOP over-expression in AECII may result in lung fibrosis, it may be

easily asked if over-expression of CHOP in ß-cells may cause diabetes, in hepatocytes liver cirrhosis and in renal tubular cells kidney fibrosis.
Differential regulation of splicing as a part of the unfolded protein response is a new and exciting observation made in this thesis. This work revealed a quite large set of more than 100 candidates for alternatively spliced mRNAs (still requiring verification by alternative techniques). Even though it can be stated that some of the affected genes have the potential to play important roles in the UPR, the actual effects of the individual events are still completely unknown and should an issue of future research.

6. REFERENCES

American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001 (2002). In: *Am J Respir Crit Care Med* 165 (2), S. 277–304.

Acosta-Alvear, D.; Zhou, Y.; Blais, A.; Tsikitis, M.; Lents, N.H; Arias, C. *et al*. (2007): XBP1 Controls Diverse Cell Type- and Condition-Specific Transcriptional Regulatory Networks. In: *Molecular Cell* 27, S. 53–66.

Adachi, Yusuke; Yamamoto, Keisuke; Okada, Tetsuya; Yoshida, Hiderou; Harada, Akihiro; Mori, Kazutoshi (2008): ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. In: *Cell Struct Funct* 33 (1), S. 75–89.

Allagnat, F.; Christulia, F.; Ortis, F.; Pirot, P.; Lortz, S.; Lenzen, S. *et al*. (2010): Sustained production of spliced X-box binding protein 1 (XBP1) induces pancreatic beta cell dysfunction and apoptosis. In: *Diabetologia* 53 (6), S. 1120–1130.

Anderson, ChristopherD; Upadhya, Gundumi; Conzen, KendraD; Jia, Jianlou; Brunt, ElizabethM; Tiriveedhi, Venkataswarup *et al*. (2011): Endoplasmic reticulum stress is a mediator of posttransplant injury in severely steatotic liver allografts. In: *Liver Transpl* 17 (2), S. 189–200.

Araki, E.; Oyadomari, S.; Mori, M. (2003): Endoplasmic Reticulum Stress and Diabetes Mellitus. In: *Internal Medicine* 42 (1), S. 7–14.

Armanios, M.Y; Chen, J.J; Cogan, J.D; Alder, J.K; Ingersoll, R.G; Markin, C. *et al*. (2007): Telomerase Mutations in Families with Idiopathic Pulmonary Fibrosis. In: *The New England journal of medicine* 356 (13), S. 1317–1326.

Ashcroft, T.; Simpson, J. M.; Timbrell, V. (1988): Simple method of estimating severity of pulmonary fibrosis on a numerical scale. In: *J Clin Pathol* 41 (4), S. 467–470.

Averous, Julien; Bruhat, Alain; Jousse, Celine; Carraro, Valerie; Thiel, Gerald; Fafournoux, Pierre (2004): Induction of CHOP expression by amino acid limitation requires both ATF4 expression and ATF2 phosphorylation. In: *J Biol Chem* 279 (7), S. 5288–5297.

Back, SungHoon; Lee, Kyungho; Vink, Elizabeth; Kaufman, RandalJ (2006): Cytoplasmic IRE1alpha-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. In: *J Biol Chem* 281 (27), S. 18691–18706.

Backer, JosephM; Krivoshein, ArcadiusV; Hamby, CarlV; Pizzonia, John; Gilbert, KennethS; Ray, YonatonS *et al*. (2009): Chaperone-targeting cytotoxin and endoplasmic reticulum stress-inducing drug synergize to kill cancer cells. In: *Neoplasia* 11 (11), S. 1165–1173.

Barbas-Filho, J. V.; Ferreira, M. A.; Sesso, A.; Kairalla, R. A.; Carvalho, C. R.; Capelozzi, V. L. (2001): Evidence of type II pneumocyte apoptosis in the pathogenesis of idiopathic pulmonary fibrosis (IFP)/usual interstitial pneumonia (UIP). In: *Journal of clinical pathology* 54 (2), S. 132–138.

Beeh, K. M.; Beier, J.; Haas, I. C.; Kornmann, O.; Micke, P.; Buhl, R. (2002): Glutathione deficiency of the lower respiratory tract in patients with idiopathic pulmonary fibrosis. In: *Eur Respir J* 19 (6), S. 1119–1123.

Benjamini, Y. Hochberg Y. (1995): the false discovery rate: a practical and powerful approach to multiple testing. In: *Journal of the Royal Statistical Society Series B,* (57), S. 289–300.

Bergeron, Anne; Jorquera, Rossana; Orejuela, Diana; Tanguay, RobertM (2006): Involvement of endoplasmic reticulum stress in hereditary tyrosinemia type I. In: *J Biol Chem* 281 (9), S. 5329–5334.

Bernales, S.; Papa, F.R; Walter, P. (2006): Intracellular Signaling by the Unfolded Protein Response. In: *Annu. Rev. Cell Dev. Biol.* 22, S. 487–508.

Brody, J. S.; Williams, M. C. (1992): Pulmonary alveolar epithelial cell differentiation. In: *Annu Rev Physiol* 54, S. 351–371.

Bromati, CarlaR; Lellis-Santos, Camilo; Yamanaka, TatianaS; Nogueira, TatianeCA; Leonelli, Mauro; Caperuto, LucianaC *et al*. (2011): UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression. In: *Am J Physiol Regul Integr Comp Physiol* 300 (1), S. R92-100.

Bruhat, A.; Jousse, C.; Carraro, V.; Reimold, A. M.; Ferrara, M.; Fafournoux, P. (2000): Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. In: *Mol Cell Biol* 20 (19), S. 7192–7204.

Cameron, H.S; Somaschini, M.; Carrera, P.; Hamvas, A.; Whitsett, J.A; Wert, S.E *et al*. (2005): A COMMON MUTATION IN THE SURFACTANT PROTEIN C GENE ASSOCIATED WITH LUNG DISEASE. In: *J Pediatr* 146, S. 370–375.

Cantin, A. M.; North, S. L.; Fells, G. A.; Hubbard, R. C.; Crystal, R. G. (1987): Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. In: *The Journal of clinical investigation* 79 (6), S. 1665–1673.

Carrasco, DanielR; Sukhdeo, Kumar; Protopopova, Marina; Sinha, Raktim; Enos, Miriam; Carrasco, DanielE *et al*. (2007): The differentiation and stress response factor XBP-1 drives multiple myeloma pathogenesis. In: *Cancer Cell* 11 (4), S. 349–360.

Castranova, V.; Rabovsky, J.; Tucker, J. H.; Miles, P. R. (1988): The alveolar type II epithelial cell: a multifunctional pneumocyte. In: *Toxicol Appl Pharmacol* 93 (3), S. 472–483.

Chen, Yi-Jiun; Tan, BertrandChin-Ming; Cheng, Ya-Yun; Chen, Jin-Shin; Lee, Sheng-Chung (2010): Differential regulation of CHOP translation by phosphorylated eIF4E under stress conditions. In: *Nucleic Acids Res* 38 (3), S. 764–777.

Cho, H. Y.; Reddy, S. P.; Yamamoto, M.; Kleeberger, S. R. (2004): The transcription factor NRF2 protects against pulmonary fibrosis. In: *Faseb J* 18 (11), S. 1258–1260.

Choi, AYoung; Choi, JiHyun; Lee, JungYeon; Yoon, Kyung-Sik; Choe, Wonchae; Ha, Joohun *et al*. (2010): Apigenin protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis. In: *Neurochem Int* 57 (2), S. 143–152.

Chua, F.; Gauldie, J.; Laurent, G.J (2005): Pulmonary Fibrosis Searching for Model Answers. In: *American journal of respiratory cell and molecular biology* 33, S. 9–13.

Cook, D.N; Brass, D.M; Schwartz, D.A (2002): A Matrix for New Ideas in Pulmonary Fibrosis. In: *Am. J. Respir. Cell Mol. Biol.* 27, S. 122–124.

Corti, M.; Brody, A.R.; Harrison, J.H (1996): Isolation and primary culture of murine alveolar type II cells. In: Am J Respir Cell Mol Biol. 14(4):309-315.

Costabel, U.; Bonella, F. (2011): Therapie der Lungenfibrose. Neue Substanzen, neue Interventionen. In: *Internist (Berl)* 52 (12), S. 1422–1428.

Costa-Mattioli, Mauro (2008): Eppendorf winner. Switching memories ON and OFF. In: *Science* 322 (5903), S. 874–875.

Costa-Mattioli, Mauro; Gobert, Delphine; Stern, Elad; Gamache, Karine; Colina, Rodney; Cuello, Claudio *et al*. (2007): eIF2alpha phosphorylation bidirectionally regulates the switch from short- to long-term synaptic plasticity and memory. In: *Cell* 129 (1), S. 195–206.

Cullinan, S. B.; Diehl, J. A. (2004): PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. In: *The Journal of biological chemistry* 279 (19), S. 20108–20117.

Cullinan, S. B.; Diehl, J. A. (2006): Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. In: *Int J Biochem Cell Biol* 38 (3), S. 317–332.

Cullinan, S. B.; Zhang, D.; Hannink, M.; Arvisais, E.; Kaufman, R. J.; Diehl, J. A. (2003): Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. In: *Molecular and cellular biology* 23 (20), S. 7198–7209.

Cybulsky, AndreyV (2010): Endoplasmic reticulum stress in proteinuric kidney disease. In: *Kidney Int* 77 (3), S. 187–193.

Dahlin, Katherine; Mager, EdwardM; Allen, Lennell; Tigue, Zachary; Goodglick, Lee; Wadehra, Madhuri; Dobbs, Leland (2004): Identification of genes differentially expressed in rat alveolar type I cells. In: *Am J Respir Cell Mol Biol* 31 (3), S. 309–316.

Dobbs, L. G.; Gonzalez, R.; Matthay, M. A.; Carter, E. P.; Allen, L.; Verkman, A. S. (1998): Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. In: *Proc Natl Acad Sci U S A* 95 (6), S. 2991–2996.

Drexler, HannesCA (2009): Synergistic apoptosis induction in leukemic cells by the phosphatase inhibitor salubrinal and proteasome inhibitors. In: *PLoS One* 4 (1), S. e4161.

Egan, J. J.; Stewart, J. P.; Hasleton, P. S.; Arrand, J. R.; Carroll, K. B.; Woodcock, A. A. (1995): Epstein-Barr virus replication within pulmonary epithelial cells in cryptogenic fibrosing alveolitis. In: *Thorax* 50 (12), S. 1234–1239.

Eizirik, DecioL; Cnop, Miriam (2010): ER stress in pancreatic beta cells: the thin red line between adaptation and failure. In: *Sci Signal* 3 (110), S. pe7.

Eizirik, DecioL; Cardozo, AlessandraK; Cnop, Miriam (2008): The role for endoplasmic reticulum stress in diabetes mellitus. In: *Endocr Rev* 29 (1), S. 42–61.

Endo, M.; Oyadomari, S.; Suga, M.; Mori, M.; Gotoh, T. (2005): The ER Stress Pathway Involving CHOP Is Activated in the Lungs of LPS-Treated Mice. In: *J. Biochem.* 138, S. 501–507.

Erbay, Ebru; Babaev, VladimirR; Mayers, JaredR; Makowski, Liza; Charles, KhanichiN; Snitow, MelindaE *et al*. (2009): Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. In: *Nat Med* 15 (12), S. 1383–1391.

Fawcett, T. W.; Martindale, J. L.; Guyton, K. Z.; Hai, T.; Holbrook, N. J. (1999): Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. In: *The Biochemical journal* 339 (Pt 1), S. 135–141.

Fehrenbach, Heinz; Zimmermann, Gregor; Starke, Ellen; Bratu, VladA; Conrad, Dominik; Yildirim, AliO; Fehrenbach, Antonia (2007): Nitrogen dioxide induces apoptosis and proliferation but not emphysema in rat lungs. In: *Thorax* 62 (5), S. 438–446.

Fonseca, SonyaG; Ishigaki, Shinsuke; Oslowski, ChristineM; Lu, Simin; Lipson, KathrynL; Ghosh, Rajarshi *et al*. (2010): Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. In: *J Clin Invest* 120 (3), S. 744–755.

Friedman, A. D. (1996): GADD153/CHOP, a DNA damage-inducible protein, reduced CAAT/enhancer binding protein activities and increased apoptosis in 32D c13 myeloid cells. In: *Cancer Res* 56 (14), S. 3250–3256.

Gachon, F.; Gaudray, G.; Thebault, S.; Basbous, J.; Koffi, J. A.; Devaux, C.; Mesnard, J. (2001): The cAMP response element binding protein-2 (CREB-2) can interact with the C/EBP-homologous protein (CHOP). In: *FEBS Lett* 502 (1-2), S. 57–62.

Gardner, LawrenceB (2008): Hypoxic inhibition of nonsense-mediated RNA decay regulates gene expression and the integrated stress response. In: *Mol Cell Biol* 28 (11), S. 3729–3741.

Geiser, Thomas (2003): Idiopathic pulmonary fibrosis--a disorder of alveolar wound repair? In: *Swiss Med Wkly* 133 (29-30), S. 405–411.

Gewandter, JenniferS; Staversky, RhondaJ; O'Reilly, MichaelA (2009): Hyperoxia augments ER-stress-induced cell death independent of BiP loss. In: *Free Radic Biol Med* 47 (12), S. 1742–1752.

Gharaee-Kermani, M.; Phan, S. H. (2005): Molecular mechanisms of and possible treatment strategies for idiopathic pulmonary fibrosis. In: *Curr Pharm Des* 11 (30), S. 3943–3971.

Gonzalez, Robert; Yang, YeeHwa; Griffin, Chandi; Allen, Lennell; Tigue, Zachary; Dobbs, Leland (2005): Freshly isolated rat alveolar type I cells, type II cells, and cultured type II cells have distinct molecular phenotypes. In: *Am J Physiol Lung Cell Mol Physiol* 288 (1), S. L179-89.

Goodall, JaneC; Wu, Changxin; Zhang, Yongsheng; McNeill, Louise; Ellis, Lou; Saudek, Vladimir; Gaston, JSHill (2010): Endoplasmic reticulum stress-induced transcription factor, CHOP, is crucial for dendritic cell IL-23 expression. In: *Proc Natl Acad Sci U S A* 107 (41), S. 17698–17703.

Gotoh, T.; Terada, l. K.; Oyadomari, S.; Mori, M. (2004): hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. In: *Cell Death and Differentiation* 11, S. 390–402.

Green, ThomasA; Alibhai, ImranN; Unterberg, Stephen; Neve, RachaelL; Ghose, Subroto; Tamminga, CarolA; Nestler, EricJ (2008): Induction of activating transcription factors (ATFs) ATF2, ATF3, and ATF4 in the nucleus accumbens and their regulation of emotional behavior. In: *J Neurosci* 28 (9), S. 2025–2032.

Groenendyk, Jody; Michalak, Marek (2005): Endoplasmic reticulum quality control and apoptosis. In: *Acta Biochim Pol* 52 (2), S. 381–395.

Guégan, C.; Przedborski, S. (2003): Programmed cell death in amyotrophic lateral sclerosis. In: *The Journal of Clinical Investigation* 111 (2), S. 153–161.

Guenet, JeanLouis; Bonhomme, Francois (2003): Wild mice: an ever-increasing contribution to a popular mammalian model. In: *Trends Genet* 19 (1), S. 24–31.

Günther, A.; Markart, P.; Eickelberg, O.; Seeger, W. (2006): [Pulmonary fibrosis--a therapeutic dilemma?]. In: *Med Klin (Munich)* 101 (4), S. 308–312.

Guo, Shui-Long; Peng, Zheng; Yang, Xue; Fan, Kai-Ji; Ye, Hui; Li, Zhen-Hua *et al.* (2011): miR-148a promoted cell proliferation by targeting p27 in gastric cancer cells. In: *Int J Biol Sci* 7 (5), S. 567–574.

Halterman, MarcW; De, JesusChristopher; Rempe, DavidA; Schor, NinaF; Federoff, HowardJ (2008): Loss of c/EBP-beta activity promotes the adaptive to apoptotic switch in hypoxic cortical neurons. In: *Mol Cell Neurosci* 38 (2), S. 125–137.

Halterman, MarcW; Gill, Molly; DeJesus, Chris; Ogihara, Mitsunori; Schor, NinaF; Federoff, HowardJ (2010): The endoplasmic reticulum stress response factor CHOP-10 protects against hypoxia-induced neuronal death. In: *J Biol Chem* 285 (28), S. 21329–21340.

Hamada, Hiromichi; Suzuki, Masashi; Yuasa, Shigeki; Mimura, Naoya; Shinozuka, Norihiro; Takada, Yuki *et al.* (2004): Dilated cardiomyopathy caused by aberrant

endoplasmic reticulum quality control in mutant KDEL receptor transgenic mice. In: *Mol Cell Biol* 24 (18), S. 8007–8017.

Han, Xue-Ji; Chae, Jei-Keon; Lee, Mi-Jin; You, Kyung-Ran; Lee, Byung-Ho; Kim, Dae-Ghon (2005): Involvement of GADD153 and cardiac ankyrin repeat protein in hypoxia-induced apoptosis of H9c2 cells. In: *J Biol Chem* 280 (24), S. 23122–23129.

Harding, H. P.; Zhang, Y.; Bertolotti, A.; Zeng, H.; Ron, D. (2000): Perk is essential for translational regulation and cell survival during the unfolded protein response. In: *Mol Cell* 5 (5), S. 897–904.

Harding, Heather P.; Calfon, Marcella; Urano, Fumihiko; Novoa, Isabel; Ron, David (2002): TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL IN THEMAMMALIAN UNFOLDED PROTEIN RESPONSE. In: *Annu. Rev. Cell Dev. Biol.* 18, S. 575–599.

Harding, HeatherP; Zhang, Yuhong; Scheuner, Donalyn; Chen, Jane-Jane; Kaufman, RandalJ; Ron, David (2009): Ppp1r15 gene knockout reveals an essential role for translation initiation factor 2 alpha (eIF2alpha) dephosphorylation in mammalian development. In: *Proc Natl Acad Sci U S A* 106 (6), S. 1832–1837.

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. In: *Molecular Biology of the Cell* 10, S. 3787–3799.

He, B. (2006): Viruses, endoplasmatic reticulum stress, and interferon responses. In: *Cell Death and Differentiation* 13, S. 393–403.

He, C. H.; Gong, P.; Hu, B.; Stewart, D.; Choi, M. E.; Choi, A. M.; Alam, J. (2001): Identification of activating transcription factor 4 (ATF4) as an Nrf2-interacting protein. Implication for heme oxygenase-1 gene regulation. In: *The Journal of biological chemistry* 276 (24), S. 20858–20865.

Healy, SandraJM; Gorman, AdrienneM; Mousavi-Shafaei, Parisa; Gupta, Sanjeev; Samali, Afshin (2009): Targeting the endoplasmic reticulum-stress response as an anticancer strategy. In: *Eur J Pharmacol* 625 (1-3), S. 234–246.

Hetz, Claudio; Glimcher, Laurie (2008): The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. In: *Trends Cell Biol* 18 (1), S. 38–44.

Hetz, Claudio; Glimcher, LaurieH (2009): Fine-tuning of the unfolded protein response: Assembling the IRE1alpha interactome. In: *Mol Cell* 35 (5), S. 551–561.

Hirose, Hidenori; Arasaki, Kohei; Dohmae, Naoshi; Takio, Koji; Hatsuzawa, Kiyotaka; Nagahama, Masami *et al*. (2004): Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. In: *EMBO J* 23 (6), S. 1267–1278.

Hong, Min; Luo, Shengzhan; Baumeister, Peter; Huang, Jen-Ming; Gogia, RaveenK; Li, Mingqing; Lee, AmyS (2004): Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response. In: *J Biol Chem* 279 (12), S. 11354–11363.

Horibe, Tomohisa; Hoogenraad, NicholasJ (2007): The chop gene contains an element for the positive regulation of the mitochondrial unfolded protein response. In: *PLoS One* 2 (9), S. e835.

Hotamisligil, GokhanS (2010): Endoplasmic reticulum stress and atherosclerosis. In: *Nat Med* 16 (4), S. 396–399.

Hoyer-Hansen, M.; Jaattela, M. (2007): Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. In: *Cell Death Differ* 14 (9), S. 1576–1582.

Huang, Ssu-Ming; Cheung, Chi-Wai; Chang, Chih-Shiang; Tang, Chih-Hsin; Liu, Ju-Fang; Lin, Yu-Hsin *et al*. (2011): Phloroglucinol derivative MCPP induces cell apoptosis in human colon cancer. In: *J Cell Biochem* 112 (2), S. 643–652.

Huang, Tao; Wan, Yigang; Zhu, Ying; Fang, Xin; Hiramatsu, Nobuhiko; Hayakawa, Konihiro *et al*. (2009): Downregulation of gap junction expression and function by endoplasmic reticulum stress. In: *J Cell Biochem* 107 (5), S. 973–983.

Jiang, Hao-Yuan; Wek, ShereeA; McGrath, BarbaraC; Lu, Dan; Hai, Tsonwin; Harding, HeatherP *et al*. (2004): Activating transcription factor 3 is integral to the eukaryotic initiation factor 2 kinase stress response. In: *Mol Cell Biol* 24 (3), S. 1365–1377.

Johnson, MeshellD; Widdicombe, JonathanH; Allen, Lennell; Barbry, Pascal; Dobbs, LelandG (2002): Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. In: *Proc Natl Acad Sci U S A* 99 (4), S. 1966–1971.

Kajstura, Jan; Rota, Marcello; Hall, SeanR; Hosoda, Toru; D'Amario, Domenico; Sanada, Fumihiro *et al*. (2011): Evidence for human lung stem cells. In: *N Engl J Med* 364 (19), S. 1795–1806.

Kammoun, HeleneL; Hainault, Isabelle; Ferre, Pascal; Foufelle, Fabienne (2009): Nutritional related liver disease: targeting the endoplasmic reticulum stress. In: *Curr Opin Clin Nutr Metab Care* 12 (6), S. 575–582.

Kapoor, Ashwani; Sanyal, ArunJ (2009): Endoplasmic reticulum stress and the unfolded protein response. In: *Clin Liver Dis* 13 (4), S. 581–590.

Kaser, Arthur; Lee, Ann-Hwee; Franke, Andre; Glickman, JonathanN; Zeissig, Sebastian; Tilg, Herbert *et al*. (2008): XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. In: *Cell* 134 (5), S. 743–756.

Kaufman, RandalJ (2002): Orchestrating the unfolded protein response in health and disease. In: *J Clin Invest* 110 (10), S. 1389–1398.

Kim IY; Kang YJ; Yoon MJ; Kim EH; Kim SU; Kwon TK *et al*. (2011): Amiodarone sensitizes human glioma cells but not astrocytes to TRAIL-induced apoptosis via CHOP-mediated DR5 upregulation. In: *Neuro Oncol* 13 (3), S. 267–279.

Kim, InKi; Shu, Chih-Wen; Xu, Wenjie; Shiau, Chung-Wai; Grant, Daniel; Vasile, Stefan *et al*. (2009): Chemical biology investigation of cell death pathways activated by endoplasmic reticulum stress reveals cytoprotective modulators of ASK1. In: *J Biol Chem* 284 (3), S. 1593–1603.

Kinnula, V. L.; Fattman, C. L.; Tan, R. J.; Oury, T. D. (2005): Oxidative stress in pulmonary fibrosis: a possible role for redox modulatory therapy. In: *American journal of respiratory and critical care medicine* 172 (4), S. 417–422.

Kinnula, V. L.; Hodgson, U. A.; Lakari, E. K.; Tan, R. J.; Sormunen, R. T.; Soini, Y. M. *et al*. (2006): Extracellular superoxide dismutase has a highly specific localization in idiopathic pulmonary fibrosis/usual interstitial pneumonia. In: *Histopathology* 49 (1), S. 66–74.

Knorre, Alexander (2001): Untersuchung der durch den Transkriptionsfaktor NF-kB vermittelten ER-Overload Response bei Zystischer Fibrose. Inaugural-Dissertation. Albert-Ludwigs-Universität, Freiburg im Breisgau. Fakultät für Biologie.

Kögel, Donat; Svensson, Birte; Copanaki, Ekaterini; Anguissola, Sergio; Bonner, Caroline; Thurow, Nadia *et al*. (2006): Induction of transcription factor CEBP homology protein mediates hypoglycaemia-induced necrotic cell death in human neuroblastoma cells. In: *J Neurochem* 99 (3), S. 952–964.

Korfei, Martina; Ruppert, Clemens; Mahavadi, Poornima; Henneke, Ingrid; Markart, Philipp; Koch, Miriam *et al*. (2008): Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. In: *Am J Respir Crit Care Med* 178 (8), S. 838–846.

Korfei, Martina; Schmitt, Sigrid; Ruppert, Clemens; Henneke, Ingrid; Markart, Philipp; Loeh, Benjamin *et al*. (2011): Comparative proteomic analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF) and lung transplant donor lungs. In: *J Proteome Res* 10 (5), S. 2185–2205.

Kozak, M. (1987): At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. In: *J Mol Biol* 196 (4), S. 947–950.

Kuang, Ersheng; Wan, Qingwen; Li, Xiaojuan; Xu, Hua; Liu, Qingzhen; Qi, Yipeng (2005): ER Ca2+ depletion triggers apoptotic signals for endoplasmic reticulum (ER) overload response induced by overexpressed reticulon 3 (RTN3/HAP). In: *J Cell Physiol* 204 (2), S. 549–559.

Kudoh, Takuya; Kimura, Junko; Lu, Zheng-Guang; Miki, Yoshio; Yoshida, Kiyotsugu (2010): D4S234E, a novel p53-responsive gene, induces apoptosis in response to DNA damage. In: *Exp Cell Res* 316 (17), S. 2849–2858.

Kuwano, K.; Kunitake, R.; Maeyama, T.; Hagimoto, N.; Kawasaki, M.; Matsuba, T. *et al*. (2001): Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. In: *American journal of physiology* 280 (2), S. L316-25.

Kuwano, K.; Nakashima, N.; Inoshima, I.; Hagimoto, N.; Fujita, M.; Yoshimi, M. *et al*. (2003): Oxidative stress in lung epithelial cells from patients with idiopathic interstitial pneumonias. In: *Eur Respir J* 21 (2), S. 232–240.

Kwok SC; Daskal I (2008): Brefeldin A activates CHOP promoter at the AARE, ERSE and AP-1 elements. In: *Mol Cell Biochem* 319 (1-2), S. 203–208.

Lai, KinWai; Koh, KingXin; Loh, Marie; Tada, Kotaro; Subramaniam, ManishMani; Lim, XnYii *et al*. (2010): MicroRNA-130b regulates the tumour suppressor RUNX3 in gastric cancer. In: *Eur J Cancer* 46 (8), S. 1456–1463.

Lassot, I.; Segeral, E.; Berlioz-Torrent, C.; Durand, H.; Groussin, L.; Hai, T. *et al*. (2001): ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase. In: *Mol Cell Biol* 21 (6), S. 2192–2202.

Lauer, MarkE; Erzurum, SerpilC; Mukhopadhyay, Durba; Vasanji, Amit; Drazba, Judith; Wang, Aimin *et al*. (2008): Differentiated murine airway epithelial cells synthesize a leukocyte-adhesive hyaluronan matrix in response to endoplasmic reticulum stress. In: *J Biol Chem* 283 (38), S. 26283–26296.

Lawson, W. E.; Crossno, P. F.; Polosukhin, V. V.; Roldan, J.; Cheng, D. S.; Lane, K. B. *et al*. (2008): Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. In: *American journal of physiology* 294 (6), S. L1119-26.

Lawson, W. E.; Grant, S. W.; Ambrosini, V.; Womble, K. E.; Dawson, E. P.; Lane, K. B. *et al*. (2004): Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. In: *Thorax* 59 (11), S. 977–980.

Lawson, WilliamE; Cheng, Dong-Sheng; Degryse, AmberL; Tanjore, Harikrishna; Polosukhin, VasiliyV; Xu, XiaochuanC *et al*. (2011): Endoplasmic reticulum stress enhances fibrotic remodeling in the lungs. In: *Proc Natl Acad Sci U S A* 108 (26), S. 10562–10567.

Lee, DoYeon; Lee, Kyu-Sun; Lee, HyunJung; Kim, DoHee; Noh, YooHun; Yu, Kweon *et al*. (2010): Activation of PERK signaling attenuates Abeta-mediated ER stress. In: *PLoS One* 5 (5), S. e10489.

Lee, K.; Tirasophon, W.; Shen, X.; Michalak, M.; Prywes, R.; Okada, T. *et al*. (2002): IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. In: *Genes Dev* 16 (4), S. 452–466.

Lemin, AndrewJ; Saleki, Khalil; van, LithMarcel; Benham, AdamM (2007): Activation of the unfolded protein response and alternative splicing of ATF6alpha in HLA-B27 positive lymphocytes. In: *FEBS Lett* 581 (9), S. 1819–1824.

Lenz, A. G.; Costabel, U.; Maier, K. L. (1996): Oxidized BAL fluid proteins in patients with interstitial lung diseases. In: *Eur Respir J* 9 (2), S. 307–312.

Lew QJ; Chu KL; Lee J; Koh PL; Rajasegaran V; Teo JY; Chao SH (2011): PCAF interacts with XBP-1S and mediates XBP-1S-dependent transcription. In: *Nucleic Acids Res* 39 (2), S. 429–439.

Li, He; Xu, Dakang; Li, Jinhua; Berndt, MichaelC; Liu, Jun-Ping (2006): Transforming growth factor beta suppresses human telomerase reverse transcriptase (hTERT) by Smad3 interactions with c-Myc and the hTERT gene. In: *J Biol Chem* 281 (35), S. 25588–25600.

Li, Yu; Xu, Shanqin; Giles, Amber; Nakamura, Kazuto; Lee, JongWoo; Hou, Xiuyun *et al*. (2011): Hepatic overexpression of SIRT1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver. In: *FASEB J* 25 (5), S. 1664–1679.

Liffers, Sven-T; Munding, JohannaB; Vogt, Markus; Kuhlmann, JanD; Verdoodt, Berlinda; Nambiar, Sandeep *et al*. (2011): MicroRNA-148a is down-regulated in

human pancreatic ductal adenocarcinomas and regulates cell survival by targeting CDC25B. In: *Lab Invest* 91 (10), S. 1472–1479.

Lin, Jiang; Wang, Ya-Li; Qian, Jun; Yao, Dong-Ming; Zhu, Zhao-Hui; Qian, Zhen; Xu, Wen-Rong (2010): Aberrant methylation of DNA-damage-inducible transcript 3 promoter is a common event in patients with myelodysplastic syndrome. In: *Leuk Res* 34 (8), S. 991–994.

Lin, JonathanH; Li, Han; Yasumura, Douglas; Cohen, HannahR; Zhang, Chao; Panning, Barbara *et al*. (2007): IRE1 signaling affects cell fate during the unfolded protein response. In: *Science* 318 (5852), S. 944–949.

Lipson, KathrynL; Fonseca, SonyaG; Urano, Fumihiko (2006): Endoplasmic reticulum stress-induced apoptosis and auto-immunity in diabetes. In: *Curr Mol Med* 6 (1), S. 71–77.

Lok, S. S.; Haider, Y.; Howell, D.; Stewart, J. P.; Hasleton, P. S.; Egan, J. J. (2002): Murine gammaherpes virus as a cofactor in the development of pulmonary fibrosis in bleomycin resistant mice. In: *Eur Respir J* 20 (5), S. 1228–1232.

Lozon, TriciaI; Altemeier, W.A (2009): CHOP Deficient Mice Have Augmented Lung Injury with Hyperoxia. In: *Am J Respir Crit Care Med*, 2009 (179).

Lozon, TriciaI; Eastman, AlisonJ; Matute-Bello, Gustavo; Chen, Peter; Hallstrand, TealS; Altemeier, WilliamA (2011): PKR-dependent CHOP induction limits hyperoxia-induced lung injury. In: *Am J Physiol Lung Cell Mol Physiol* 300 (3), S. L422-9.

Ma, Stephanie; Tang, KwanHo; Chan, YuenPiu; Lee, TerenceK; Kwan, PakShing; Castilho, Antonia *et al*. (2010): miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. In: *Cell Stem Cell* 7 (6), S. 694–707.

Ma, Y.; Brewer, J. W.; Diehl, J. A.; Hendershot, L. M. (2002): Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. In: *Journal of molecular biology* 318 (5), S. 1351–1365.

Ma, Yanjun; Hendershot, LindaM (2003): Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. In: *J Biol Chem* 278 (37), S. 34864–34873.

Magne, Laurent; Blanc, Etienne; Legrand, Beatrice; Lucas, Daniele; Barouki, Robert; Rouach, Helene; Garlatti, Michele (2011): ATF4 and the integrated stress response are induced by ethanol and cytochrome P450 2E1 in human hepatocytes. In: *J Hepatol* 54 (4), S. 729–737.

Mak, BaldwinC; Wang, Qin; Laschinger, Carol; Lee, Wilson; Ron, David; Harding, HeatherP *et al*. (2008): Novel function of PERK as a mediator of force-induced apoptosis. In: *J Biol Chem* 283 (34), S. 23462–23472.

Malhotra, J. D.; Miao, H.; Zhang, K.; Wolfson, A.; Pennathur, S.; Pipe, S. W.; Kaufman, R. J. (2008): Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. In: *Proceedings of the National Academy of Sciences of the United States of America* 105 (47), S. 18525–18530.

Markart, P.; Ruppert, C.; Wygrecka, M.; Schmidt, R.; Korfei, M.; Harbach, H. *et al*. (2007): Surfactant protein C mutations in sporadic forms of idiopathic interstitial pneumonias. In: *Eur Respir J* 29 (1), S. 134–137.

Markart, P.; Seeger, W.; Gunther, A. (2006): [Differential therapy of pulmonary fibrosis.]. In: *Der Internist*.

Matsumoto, M.; Minami, M.; Takeda, K.; Sakao, Y.; Akira, S. (1996): Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. In: *FEBS Lett* 395 (2-3), S. 143–147.

Mimura, N.; Hamada, H.; Kashio, M.; Jin, H.; Toyama, Y.; Kimura, K. *et al*. (2007): Aberrant quality control in the endoplasmic reticulum impairs the biosynthesis of pulmonary surfactant in mice expressing mutant BiP. In: *Cell Death Differ* 14 (8), S. 1475–1485.

Montaldo, C.; Cannas, E.; Ledda, M.; Rosetti, L.; Congiu, L.; Atzori, L. (2002): Bronchoalveolar glutathione and nitrite/nitrate in idiopathic pulmonary fibrosis and sarcoidosis. In: *Sarcoidosis Vasc Diffuse Lung Dis* 19 (1), S. 54–58.

Morse, Elizabeth; Schroth, Jana; You, Young-Hyun; Pizzo, DonaldP; Okada, Shinichi; Ramachandrarao, Satish *et al*. (2010): TRB3 is stimulated in diabetic kidneys, regulated by the ER stress marker CHOP, and is a suppressor of podocyte MCP-1. In: *Am J Physiol Renal Physiol* 299 (5), S. F965-72.

Mulugeta, S.; Maguire, J.A; Newitt, J.L; Russo, S.J; Kotorashvili, A.; Beers, M.F (2007): Misfolded BRICHOS SP-C mutant proteins induce apoptosis via caspase 4 and cytochrome c related mechanisms. In: *American journal of physiology* 32 (6), S. 521–530.

Myers, J. L.; Katzenstein, A. L. (1988): Epithelial necrosis and alveolar collapse in the pathogenesis of usual interstitial pneumonia. In: *Chest* 94 (6), S. 1309–1311.

Myers, J.L; Selman, M. (2004): Respiratory Epithelium in Usual Interstitial Pneumonia/Idiopathic Pulmonary Fibrosis Spark or Destructive Flame? In: *AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE* 169.

Nadanaka, S.; Yoshida, H.; Kano, F.; Murata, M.; Mori, K. (2004): Activation of Mammalian Unfolded Protein Response Is Compatible with the Quality Control System Operating in the Endoplasmic Reticulum. In: *Molecular Biology of the Cell* 15, S. 2537–2548.

Naidoo, Nirinjini (2009): ER and aging-Protein folding and the ER stress response. In: *Ageing Res Rev* 8 (3), S. 150–159.

Nakagawa, T.; Zhu, H.; Morishima, N.; Li, E.; Xu, J.; Yankner, B. A.; Yuan, J. (2000): Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. In: *Nature* 403 (6765), S. 98–103.

Namba, Takushi; Tanaka, Ken-Ichiro; Ito, Yosuke; Ishihara, Tomoaki; Hoshino, Tatsuya; Gotoh, Tomomi *et al*. (2009): Positive role of CCAAT/enhancer-binding protein homologous protein, a transcription factor involved in the endoplasmic reticulum stress response in the development of colitis. In: *Am J Pathol* 174 (5), S. 1786–1798.

Ni, Min; Zhou, Hui; Wey, Shiuan; Baumeister, Peter; Lee, AmyS (2009): Regulation of PERK signaling and leukemic cell survival by a novel cytosolic isoform of the UPR regulator GRP78/BiP. In: *PLoS One* 4 (8), S. e6868.

Nogee, L. M.; Dunbar, A. E. 3rd; Wert, S. E.; Askin, F.; Hamvas, A.; Whitsett, J. A. (2001): A mutation in the surfactant protein C gene associated with familial interstitial lung disease. In: *The New England journal of medicine* 344 (8), S. 573–579.

Novoa, I.; Zeng, H.; Harding, H.P; Ron, D. (2001): Feedback Inhibition of the Unfolded Protein response by GADD34-mediated Dephosphorylation of eIF2a. In: *The Journal of Cell Biology* 153 (5), S. 1011–1021.

Nozaki S; Sledge Jr GW; Nakshatri H (2001): Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death. In: *Oncogene* 20 (17), S. 2178–2185.

Oakes, S.A; Lin, S.S; Bassik, M.C (2006): The Control of Endoplasmic Reticulum-Initiated Apoptosis by the BCL-2 Family of Proteins. In: *Current Molecular Medicine* 6, S. 99–109.

Oda, Yukako; Okada, Tetsuya; Yoshida, Hiderou; Kaufman, RandalJ; Nagata, Kazuhiro; Mori, Kazutoshi (2006): Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. In: *J Cell Biol* 172 (3), S. 383–393.

Oh, You-Take; Liu, Xiangguo; Yue, Ping; Kang, Sumin; Chen, Jing; Taunton, Jack *et al*. (2010): ERK/ribosomal S6 kinase (RSK) signaling positively regulates death receptor 5 expression through co-activation of CHOP and Elk1. In: *J Biol Chem* 285 (53), S. 41310–41319.

Ohtsuki T; Kikuchi H; Koyano T; Kowithayakorn T; Sakai T; Ishibashi M (2009): Death receptor 5 promoter-enhancing compounds isolated from Catimbium speciosum and their enhancement effect on TRAIL-induced apoptosis. In: *Bioorg Med Chem* 17 (18), S. 6748–6754.

Oikawa, Daisuke; Kimata, Yukio; Kohno, Kenji (2007): Self-association and BiP dissociation are not sufficient for activation of the ER stress sensor Ire1. In: *J Cell Sci* 120 (Pt 9), S. 1681–1688.

Oikonomou, N.; Thanasopoulou, A.; Tzouvelekis, A.; Harokopos, V.; Paparountas, T.; Nikitopoulou, I. *et al*. (2009): Gelsolin expression is necessary for the development of modelled pulmonary inflammation and fibrosis. In: *Thorax* 64 (6), S. 467–475.

Oliveira, SusanaJ; Pinto, JorgeP; Picarote, Goncalo; Costa, VeraM; Carvalho, Felix; Rangel, Maria *et al*. (2009): ER stress-inducible factor CHOP affects the expression of hepcidin by modulating C/EBPalpha activity. In: *PLoS One* 4 (8), S. e6618.

Ord, Daima; Ord, Tonis (2003): Mouse NIPK interacts with ATF4 and affects its transcriptional activity. In: *Exp Cell Res* 286 (2), S. 308–320.

Oslowski, ChristineM; Urano, Fumihiko (2010): The binary switch between life and death of endoplasmic reticulum-stressed beta cells. In: *Curr Opin Endocrinol Diabetes Obes* 17 (2), S. 107–112.

Oyadomari, S.; Mori, M. (2004): Roles of CHOP/GADD153 in endoplasmic reticulum stress. In: *Cell Death Differ* 11 (4), S. 381–389.

Oyadomari, S.; Takeda, K.; Takiguchi, M.; Gotoh, T.; Matsumoto, M.; Wada, I. *et al*. (2001): Nitric oxide-induced apoptosis in pancreatic b cells is mediated by the endoplasmic reticulum stress pathway. In: *PNAS* 98 (19), S. 10845–10850.

Oyadomari, Seiichi; Koizumi, Akio; Takeda, Kiyoshi; Gotoh, Tomomi; Akira, Shizuo; Araki, Eiichi; Mori, Masataka (2002): Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. In: *J Clin Invest* 109 (4), S. 525–532.

Palam LR; Baird TD; Wek RC (2011): Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. In: *J Biol Chem*.

Pallet, Nicolas; Bouvier, Nicolas; Beaune, Philippe; Legendre, Christophe; Anglicheau, Dany; Thervet, Eric (2010): Implication du stress du reticulum endoplasmique en transplantation d'organe solide. In: *Med Sci (Paris)* 26 (4), S. 397–403.

Pardo, A.; Selman, M. (2002): Idiopathic pulmonary fibrosis: new insights in its pathogenesis. In: *The International Journal of Biochemistry & Cell Biology* 34, S. 1534–1538.

Park, GaBin; Kim, YeongSeok; Lee, Hyun-Kyung; Song, Hyunkeun; Cho, Dae-Ho; Lee, WangJae; Hur, DaeYoung (2010): Endoplasmic reticulum stress-mediated apoptosis of EBV-transformed B cells by cross-linking of CD70 is dependent upon generation of reactive oxygen species and activation of p38 MAPK and JNK pathway. In: *J Immunol* 185 (12), S. 7274–7284.

Pereira, RenataC; Stadmeyer, LisaE; Smith, DeannaL; Rydziel, Sheila; Canalis, Ernesto (2007): CCAAT/Enhancer-binding protein homologous protein (CHOP) decreases bone formation and causes osteopenia. In: *Bone* 40 (3), S. 619–626.

Poornima Mahavadi (2009): Role of impaired lysosomal trafficking in the Role of impaired lysosomal trafficking in the development of lung fibrosis in a murine model of Hermansky-Pudlak syndrome. Inaugural - Dissertation. Justus-Liebig-Universität Gießen, Gießen.

Puri, Puneet; Mirshahi, Faridoddin; Cheung, Onpan; Natarajan, Ramesh; Maher, JamesW; Kellum, JohnM; Sanyal, ArunJ (2008): Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. In: *Gastroenterology* 134 (2), S. 568–576.

Puzianowska-Kuznicka, Monika; Kuznicki, Jacek (2009): The ER and ageing II: calcium homeostasis. In: *Ageing Res Rev* 8 (3), S. 160–172.

R Development Core Team (2011): R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Razzaque, M.S; Taguchi, T. (2003): Pulmonary fibrosis: Cellular and molecular events. In: *Pathology International* 53 (133-145).

Reneker, LixingW; Chen, Huiyi; Overbeek, PaulA (2011): Activation of unfolded protein response in transgenic mouse lenses. In: *Invest Ophthalmol Vis Sci* 52 (5), S. 2100–2108.

Rio, D. C. (1993): Splicing of pre-mRNA: mechanism, regulation and role in development. In: *Curr Opin Genet Dev* 3 (4), S. 574–584.

Ron, D. (2002): Translational control in the endoplasmic reticulum stress response. In: *J. Clin. Invest.* 110 (10), S. 1383–1388.

Ron, David; Oyadomari, Seiichi (2004): Lipid phase perturbations and the unfolded protein response. In: *Dev Cell* 7 (3), S. 287–288.

Roy, B.; Lee, A.S (1999): The mammalian endoplasmic reticulum stress response element consists of an evolutionary conserved tripartite structure and interacts with a novel stress-inducible complex. In: *Nucleic Acids Res.* 27 (6), S. 1437–1443.

Roper, J.M.; Staversky, R.J.; Finkelstein, J.N.; Keng, P.C.; O'Reilly, M.A (2003) : Identification and isolation of mouse type II cells on the basis of intrinsic expression of enhanced green fluorescent protein. In: *Am J Physiol Lung Cell Mol Physiol* 285 (3): S. 691–700.

Rutishauser, J.; Spiess, M. (2002): Endoplasmic reticulum storage diseases. In: *SWISS MED WKLY* 132, S. 211–222.

Rutkowski, DThomas; Arnold, StaceyM; Miller, CoreyN; Wu, Jun; Li, Jack; Gunnison, KathrynM *et al.* (2006): Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. In: *PLoS Biol* 4 (11), S. e374.

Saito, Shoichi; Takahashi, Senye; Takagaki, Nobumasa; Hirose, Tohru; Sakai, Toshiyuki (2003): 15-Deoxy-Delta(12,14)-prostaglandin J2 induces apoptosis through activation of the CHOP gene in HeLa cells. In: *Biochem Biophys Res Commun* 311 (1), S. 17–23.

Saleh, D.; Barnes, P. J.; Giaid, A. (1997): Increased production of the potent oxidant peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. In: *American journal of respiratory and critical care medicine* 155 (5), S. 1763–1769.

Salminen, Antero; Kauppinen, Anu; Suuronen, Tiina; Kaarniranta, Kai; Ojala, Johanna (2009): ER stress in Alzheimer's disease: a novel neuronal trigger for inflammation and Alzheimer's pathology. In: *J Neuroinflammation* 6, S. 41.

Sandhir, Rajat; Berman, NancyEJ (2010): Age-dependent response of CCAAT/enhancer binding proteins following traumatic brain injury in mice. In: *Neurochem Int* 56 (1), S. 188–193.

Satoh, T.; Abiru, N.; Kobayashi, M.; Zhou, H.; Nakamura, K.; Kuriya, G. *et al.* (2011): CHOP deletion does not impact the development of diabetes but suppresses the early production of insulin autoantibody in the NOD mouse. In: *Apoptosis* 16 (4), S. 438–448.

Sayer, JohnA; Otto, EdgarA; O'Toole, JohnF; Nurnberg, Gudrun; Kennedy, MichaelA; Becker, Christian *et al*. (2006a): The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. In: *Nat Genet* 38 (6), S. 674–681.

Sayer, JohnA; Otto, EdgarA; O'Toole, JohnF; Nurnberg, Gudrun; Kennedy, MichaelA; Becker, Christian *et al*. (2006b): The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. In: *Nat Genet* 38 (6), S. 674–681.

Scheuner, Donalyn; Patel, Rupali; Wang, Feng; Lee, Kuei; Kumar, Kotlo; Wu, Jun *et al*. (2006): Double-stranded RNA-dependent protein kinase phosphorylation of the alpha-subunit of eukaryotic translation initiation factor 2 mediates apoptosis. In: *J Biol Chem* 281 (30), S. 21458–21468.

Schmidt, R.; Meier, U.; Markart, P.; Grimminger, F.; Velcovsky, H. G.; Morr, H. *et al*. (2002): Altered fatty acid composition of lung surfactant phospholipids in interstitial lung disease. In: *American journal of physiology* 283 (5), S. L1079-85.

Schmittgen, T. D.; Zakrajsek, B. A. (2000): Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. In J Biochem Biophys Methods 46 (1-2), pp. 69–81.

Schröder, M.; Kaufman, R.J (2005): THEMAMMALIAN UNFOLDED PROTEIN RESPONSE. In: *Annu. Rev. Biochem.* 74, S. 739–789.

Selman, M.; Pardo, A. (2006): Role of Epithelial Cells in Idiopathic Pulmonary Fibrosis From Innocent Targets to Serial Killers. In: *Proceedings of the American Thoracic Society* 3, S. 364–372.

Selman, M.; King Jr., T.E; Pardo, A. (2001): Idiopathic Pulmonary Fibrosis: Prevailing and Evolving Hypotheses about Its Pathogenesis and Implications for Therapy. In: *Ann Intern Med.* 134, S. 136–151.

Shang, J.; Lehrman, M.A (2004): Discordance of UPR signaling by ATF6 and Ire1p-XBP1 with levels of target transcripts. In: *Biochemical and Biophysical Research Communications* 317, S. 390–396.

Shao C; Lawrence MC; Cobb MH (2010): Regulation of CCAAT/enhancer-binding protein homologous protein (CHOP) expression by interleukin-1 beta in pancreatic beta cells. In: *J Biol Chem* 285 (26), S. 19710–19719.

Sharpe, LauraJ; Luu, Winnie; Brown, AndrewJ (2011): Akt phosphorylates Sec24: new clues into the regulation of ER-to-Golgi trafficking. In: *Traffic* 12 (1), S. 19–27.

Shechtman, CarynF; Henneberry, AnnetteL; Seimon, TracieA; Tinkelenberg, ArthurH; Wilcox, LisaJ; Lee, Eunjee *et al*. (2011): Loss of subcellular lipid transport due to ARV1 deficiency disrupts organelle homeostasis and activates the unfolded protein response. In: *J Biol Chem* 286 (14), S. 11951–11959.

Shen, J.; Prywes, R. (2005): ER stress signaling by regulated proteolysis of ATF6. In: *Methods* 35, S. 382–389.

Shen, J.; Snapp, E.L; Lippincott-Schwartz, J.; Prywes, R. (2005): Stable Binding of ATF6 to BiP in the Endoplasmic Reticulum Stress Response. In: *MOLECULAR AND CELLULAR BIOLOGY* 25 (3), S. 921–932.

Shimasaki, Shogo; Koga, Tomoaki; Shuto, Tsuyoshi; Suico, MaryAnn; Sato, Takashi; Watanabe, Kenji *et al*. (2010): Endoplasmic reticulum stress increases the expression and function of toll-like receptor-2 in epithelial cells. In: *Biochem Biophys Res Commun* 402 (2), S. 235–240.

Sisson, ThomasH; Mendez, Michael; Choi, Karen; Subbotina, Natalya; Courey, Anthony; Cunningham, Andrew *et al*. (2010): Targeted injury of type II alveolar epithelial cells induces pulmonary fibrosis. In: *Am J Respir Crit Care Med* 181 (3), S. 254–263.

Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D. *et al*. (1985): Measurement of protein using bicinchoninic acid. In: *Anal Biochem* 150 (1), S. 76–85.

Smyth, G. K. (2005): Limma: linear models for microarray data. In: 'Bioinformatics and Computational Biology Solutions using R and Bioconductor. In: *Springer, New York*, S. 397--420.

Sriburi, R.; Jackowski, S.; Mori, K.; Brewer, J.W (2004): XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. In: *The Journal of Cell Biology* 167 (1).

Steele, M. P.; Speer, M. C.; Loyd, J. E.; Brown, K. K.; Herron, A.; Slifer, S. H. *et al*. (2005): Clinical and pathologic features of familial interstitial pneumonia. In: *Am J Respir Crit Care Med* 172 (9), S. 1146–1152.

Su, Nan; Kilberg, MichaelS (2008): C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene. In: *J Biol Chem* 283 (50), S. 35106–35117.

Suresh, Sukanya; McCallum, Lynn; Lu, Wanhua; Lazar, Noureddine; Perbal, Bernard; Irvine, AlexandraE (2011): MicroRNAs 130a/b are regulated by BCR-ABL and downregulate expression of CCN3 in CML. In: *J Cell Commun Signal* 5 (3), S. 183–191.

Szegezdi, E.; Logue, S.E; Gorman, A.M; Samali, A. (2006): Mediators of endoplasmic reticulum stress-induced apoptosis. In: *EMBO J.* 7 (9).

Tabas, Ira (2009): Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. In: *Antioxid Redox Signal* 11 (9), S. 2333–2339.

Takada, Koji; Hirose, Jun; Senba, Kei; Yamabe, Soichiro; Oike, Yuichi; Gotoh, Tomomi; Mizuta, Hiroshi (2011): Enhanced apoptotic and reduced protective response in chondrocytes following endoplasmic reticulum stress in osteoarthritic cartilage. In: *Int J Exp Pathol* 92 (4), S. 232–242.

Taketo, M.; Schroeder, A. C.; Mobraaten, L. E.; Gunning, K. B.; Hanten, G.; Fox, R. R. *et al*. (1991): FVB/N: an inbred mouse strain preferable for transgenic analyses. In: *Proc Natl Acad Sci U S A* 88 (6), S. 2065–2069.

Tang, Y. W.; Johnson, J. E.; Browning, P. J.; Cruz-Gervis, R. A.; Davis, A.; Graham, B. S. *et al*. (2003): Herpesvirus DNA is consistently detected in lungs of

patients with idiopathic pulmonary fibrosis. In: *J Clin Microbiol* 41 (6), S. 2633–2640.

Teodoro, T.; Odisho, T.; Sidorova, E.; Volchuk, A. (2011): PANCREATIC beta-CELLS DEPEND ON BASAL EXPRESSION OF ACTIVE ATF6alpha-p50 FOR CELL SURVIVAL EVEN UNDER NON-STRESS CONDITIONS. In: *Am J Physiol Cell Physiol*.

Tessel, MichaelA; Benham, AshleyL; Krett, NancyL; Rosen, StevenT; Gunaratne, PreethiH (2011): Role for microRNAs in regulating glucocorticoid response and resistance in multiple myeloma. In: *Horm Cancer* 2 (3), S. 182–189.

Thannickal, V. J.; Horowitz, J. C. (2006): Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. In: *Proceedings of the American Thoracic Society* 3 (4), S. 350–356.

Thannickal, V.J; Toews, G.B; White, E.S; Lynch, J.P; Martinez, F.J (2004): MECHANISMS OF PULMONARY FIBROSIS. In: *Annu. Rev. Med.* 55, S. 395–417.

Thomas, ChristophorosG; Spyrou, Giannis (2009): ERdj5 sensitizes neuroblastoma cells to endoplasmic reticulum stress-induced apoptosis. In: *J Biol Chem* 284 (10), S. 6282–6290.

Thomas, Monzy; George, NysiaI; Saini, UpasanaT; Patterson, TuckerA; Hanig, JosephP; Bowyer, JohnF (2010): Endoplasmic reticulum stress responses differ in meninges and associated vasculature, striatum, and parietal cortex after a neurotoxic amphetamine exposure. In: *Synapse* 64 (8), S. 579–593.

Tichelaar, J. W.; Lu, W.; Whitsett, J. A. (2000): Conditional expression of fibroblast growth factor-7 in the developing and mature lung. In: *J Biol Chem* 275 (16), S. 11858–11864.

Tsukamoto, K.; Hayakawa, H.; Sato, A.; Chida, K.; Nakamura, H.; Miura, K. (2000): Involvement of Epstein-Barr virus latent membrane protein 1 in disease progression in patients with idiopathic pulmonary fibrosis. In: *Thorax* 55 (11), S. 958–961.

Ubeda, M.; Habener, J. F. (2000): CHOP gene expression in response to endoplasmic-reticular stress requires NFY interaction with different domains of a conserved DNA-binding element. In: *Nucleic Acids Res* 28 (24), S. 4987–4997.

Uhal, B. D.; Gidea, C.; Bargout, R.; Bifero, A.; Ibarra-Sunga, O.; Papp, M. *et al*. (1998a): Captopril inhibits apoptosis in human lung epithelial cells: a potential antifibrotic mechanism. In: *The American journal of physiology* 275 (5 Pt 1), S. L1013-7.

Uhal, B. D.; Joshi, I.; Hughes, W. F.; Ramos, C.; Pardo, A.; Selman, M. (1998b): Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. In: *Am J Physiol* 275 (6 Pt 1), S. L1192-9.

Ulianich, Luca; Garbi, Corrado; Treglia, AntonellaSonia; Punzi, Dario; Miele, Claudia; Raciti, GregoryAlexander *et al*. (2008): ER stress is associated with dedifferentiation and an epithelial-to-mesenchymal transition-like phenotype in PC Cl3 thyroid cells. In: *J Cell Sci* 121 (Pt 4), S. 477–486.

van der Kallen, Carla J. H.; van Greevenbroek, Marleen M.; Stehouwer, Coen D.; Schalkwijk, Casper G. (2009): Endoplasmic reticulum stress-induced apoptosis in the development of diabetes: is there a role for adipose tissue and liver? In: *Apoptosis* 14 (12), S. 1424–1434.

van der Vlies, D.; Woudenberg, J.; Post, J.A (2003): Protein oxidation in aging: endoplasmic reticulum as a target. In: *Amino Acids* 25, S. 397–407.

Verma, S.; Slutsky, A.S (2007): Idiopathic Pulmonary Fibrosis — New Insights. In: *The New England journal of medicine* 356 (13), S. 1370–1372.

Vij, Neeraj; Fang, Shengyun; Zeitlin, PamelaL (2006): Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. In: *J Biol Chem* 281 (25), S. 17369–17378.

Wang YL; Qian J; Lin J; Yao DM; Qian Z; Zhu ZH; Li JY (2010): Methylation status of DDIT3 gene in chronic myeloid leukemia. In: *J Exp Clin Cancer Res* 29, S. 54.

Wang, Feng-Ming; Chen, Yi-Jiun; Ouyang, Hong-Jiao (2010): Regulation of unfolded protein response modulator XBP1s by acetylation and deacetylation. In: *Biochem J* 433 (1), S. 245–252.

Wang, Lu; Wang, Lingxian; Song, Ran; Shen, Yan; Sun, Yang; Gu, Yanhong *et al*. (2011a): Targeting sarcoplasmic/endoplasmic reticulum Ca(2)+-ATPase 2 by curcumin induces ER stress-associated apoptosis for treating human liposarcoma. In: *Mol Cancer Ther* 10 (3), S. 461–471.

Wang, R.; Ibarra-Sunga, O.; Verlinski, L.; Pick, R.; Uhal, B. D. (2000): Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. In: *American journal of physiology* 279 (1), S. L143-51.

Wang, Xin; Shi, Qi; Xu, Kun; Gao, Chen; Chen, Cao; Li, Xiao-Li *et al*. (2011b): Familial CJD associated PrP mutants within transmembrane region induced Ctm-PrP retention in ER and triggered apoptosis by ER stress in SH-SY5Y cells. In: *PLoS One* 6 (1), S. e14602.

Wang, Yongyu; Kuan, PhillipJ; Xing, Chao; Cronkhite, JenniferT; Torres, Fernando; Rosenblatt, RandallL *et al*. (2009): Genetic defects in surfactant protein A2 are associated with pulmonary fibrosis and lung cancer. In: *Am J Hum Genet* 84 (1), S. 52–59.

White, E.S; Lazar, M.H; Thannickal, V.J (2003): Pathogenetic mechanisms in usual interstitial pneumonia/idiopathic pulmonary fibrosis. In: *J Pathol* 201, S. 343–354.

Wikenheiser, K. A.; Vorbroker, D. K.; Rice, W. R.; Clark, J. C.; Bachurski, C. J.; Oie, H. K.; Whitsett, J. A. (1993): Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. In: *Proc Natl Acad Sci U S A* 90 (23), S. 11029–11033.

Wiley, JesseC; Meabon, JamesS; Frankowski, Harald; Smith, EliseA; Schecterson, LeslayannC; Bothwell, Mark; Ladiges, WarrenC (2010): Phenylbutyric acid rescues endoplasmic reticulum stress-induced suppression of

APP proteolysis and prevents apoptosis in neuronal cells. In: *PLoS One* 5 (2), S. e9135.

Wiseman, RLuke; Balch, WilliamE (2005): A new pharmacology--drugging stressed folding pathways. In: *Trends Mol Med* 11 (8), S. 347–350.

Woo, ConnieW; Cui, Dongying; Arellano, Jerry; Dorweiler, Bernhard; Harding, Heather; Fitzgerald, KatherineA *et al*. (2009): Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signalling. In: *Nat Cell Biol* 11 (12), S. 1473–1480.

Wootton, S.C.; Kim, D.S.; Kondoh, Y.; Chen, E.; Lee, J.S.; Song, J.W.; Huh, J.W.; Taniguchi, H.; Chiu, C.; Boushey, H.; Lancaster, L.H.; Wolters, P.J.; DeRisi, J.; Ganem, D.; Collard, H.R. (2011): Viral infection in acute exacerbation of idiopathic pulmonary fibrosis. In: *Am J Respir Crit Care Med* 183(12), S. 1698-1702

Wu, Jin; Zhang, Ruihua; Torreggiani, Massimo; Ting, Adrian; Xiong, Huabao; Striker, GaryE *et al*. (2010): Induction of diabetes in aged C57B6 mice results in severe nephropathy: an association with oxidative stress, endoplasmic reticulum stress, and inflammation. In: *Am J Pathol* 176 (5), S. 2163–2176.

Xin, Wei; Li, Xiaoying; Lu, Xiaochun; Niu, Kun; Cai, Jimei (2011): Involvement of endoplasmic reticulum stress-associated apoptosis in a heart failure model induced by chronic myocardial ischemia. In: *Int J Mol Med* 27 (4), S. 503–509.

Xu, C.; Bailly-Maitre, B.; Reed, J. C. (2005): Endoplasmic reticulum stress: cell life and death decisions. In: *J. Clin. Invest.* 115, S. 2656–2664.

Yamaguchi, Yukihiro; Larkin, Dennis; Lara-Lemus, Roberto; Ramos-Castaneda, Jose; Liu, Ming; Arvan, Peter (2008): Endoplasmic reticulum (ER) chaperone regulation and survival of cells compensating for deficiency in the ER stress response kinase, PERK. In: *J Biol Chem* 283 (25), S. 17020–17029.

Yamamoto, K.; Yoshida, H.; Kokame, K.; Kaufman, R.J; Mori, K. (2004): Differential Contributions of ATF6 and XBP1 to the Activation of Endoplasmic Reticulum Stress-Responsive cis-Acting Elements ERSE, UPRE and ERSE-II. In: *J. Biochem.* 136, S. 343–350.

Yamazaki T; Ohmi A; Kurumaya H; Kato K; Abe T; Yamamoto H *et al*. (2010): Regulation of the human CHOP gene promoter by the stress response transcription factor ATF5 via the AARE1 site in human hepatoma HepG2 cells. In: *Life Sci* 87 (9-10), S. 294–301.

Ye, J.; Rawson, R.B; Komuro, R.; Chen, X.; Dave, U.P; Prywes, R. *et al*. (2000): ER Stress Induces Cleavage of Membrane-Bound ATF6 by the Same Proteases that Process SREBPs. In: *Molecular Cell* 6, S. 1355–1364.

Yip, Linwah; Kelly, Lindsey; Shuai, Yongli; Armstrong, MichaeleJ; Nikiforov, YuriE; Carty, SallyE; Nikiforova, MarinaN (2011): MicroRNA signature distinguishes the degree of aggressiveness of papillary thyroid carcinoma. In: *Ann Surg Oncol* 18 (7), S. 2035–2041.

Yorimitsu, Tomohiro; Nair, Usha; Yang, Zhifen; Klionsky, DanielJ (2006): Endoplasmic reticulum stress triggers autophagy. In: *J Biol Chem* 281 (40), S. 30299–30304.

Yoshida, H. (2007): ER stress and diseases. In: *The FEBS journal* 274 (3), S. 630–658.

Yoshida, H.; Matsui, T.; Yamamoto, A.; Okada, T.; Mori, K. (2001): XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. In: *Cell* 107, S. 881–891.

Yoshida, H.; Okada, T.; Haze, K.; Yanagi, H.; Yura, T.; Negishi, M.; Mori, K. (2000): ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. In: *Mol Cell Biol* 20 (18), S. 6755–6767.

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. In: *The Journal of Cell Biology* 172 (4), S. 565–575.

Yoshida, Toshinori; Mett, Igor; Bhunia, AnilK; Bowman, Joel; Perez, Mario; Zhang, Li *et al*. (2010): Rtp801, a suppressor of mTOR signaling, is an essential mediator of cigarette smoke-induced pulmonary injury and emphysema. In: *Nat Med* 16 (7), S. 767–773.

Yu, VionnieWC; Ambartsoumian, Gourgen; Verlinden, Lieve; Moir, JanetM; Prud'homme, Josee; Gauthier, Claude *et al*. (2005): FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. In: *J Cell Biol* 169 (4), S. 591–601.

Zglinicki, T. von (2002): Oxidative stress shortens telomeres. In: *Trends Biochem Sci* 27 (7), S. 339–344.

Zhang, H.; Li, Y.; Huang, Q.; Ren, X.; Hu, H.; Sheng, H.; Lai, M. (2011): MiR-148a promotes apoptosis by targeting Bcl-2 in colorectal cancer. In: *Cell Death Differ* 18 (11), S. 1702–1710.

Zhang, K.; Kaufman, R.J (2004): Signaling the Unfolded Protein Response from the Endoplasmic Reticulum. In: *THE JOURNAL OF BIOLOGICAL CHEMISTRY* 279 (25), S. 25935–25938.

Zhang, Kezhong; Kaufman, RandalJ (2003): Unfolding the toxicity of cholesterol. In: *Nat Cell Biol* 5 (9), S. 769–770.

Zhang, Kezhong; Kaufman, RandalJ (2008): From endoplasmic-reticulum stress to the inflammatory response. In: *Nature* 454 (7203), S. 455–462.

Zhang, Kezhong; Wong, HettyN; Song, Benbo; Miller, CoreyN; Scheuner, Donalyn; Kaufman, RandalJ (2005): The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis. In: *J Clin Invest* 115 (2), S. 268–281.

Zhao, L.; Ackerman, S.L (2006): Endoplasmic reticulum stress in health and disease. In: *Current Opinion in Cell Biology* 18, S. 444–452.

Zheng, Biqiang; Liang, Linhui; Wang, Chunmeng; Huang, Shenglin; Cao, Xi; Zha, Ruopeng *et al*. (2011): MicroRNA-148a Suppresses Tumor Cell Invasion and Metastasis by Downregulating ROCK1 in Gastric Cancer. In: *Clin Cancer Res* 17 (24), S. 7574–7583.

Zhong, Q.; Zhou, B.; Ann, D. K.; Minoo, P.; Liu, Y.; Banfalvi, A. *et al*. (2010): Role of ER Stress in EMT of Alveolar Epithelial Cells: Effects of Misfolded Surfactant Protein. In: *Am J Respir Cell Mol Biol*.

Zhou, Jiahai; Liu, ChuanYin; Back, SungHoon; Clark, RobertL; Peisach, Daniel; Xu, Zhaohui; Kaufman, RandalJ (2006): The crystal structure of human IRE1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. In: *Proc Natl Acad Sci U S A* 103 (39), S. 14343–14348.

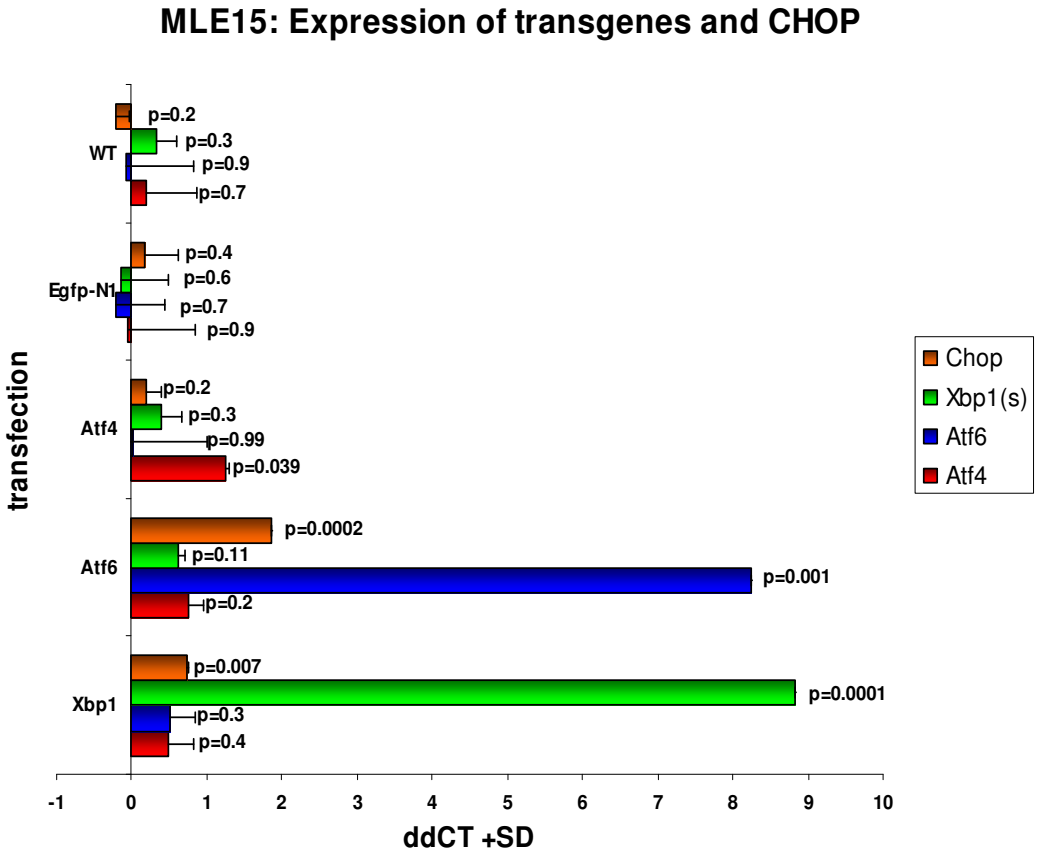
Zhou, Xin; Zhao, Fang; Wang, Zhen-Ning; Song, Yong-Xi; Chang, Hua; Chiang, Yeunpo; Xu, Hui-Mian (2012): Altered expression of miR-152 and miR-148a in ovarian cancer is related to cell proliferation. In: *Oncol Rep* 27 (2), S. 447–454.

Zhu, T.; Lobie, P. E. (2000): Janus kinase 2-dependent activation of p38 mitogen-activated protein kinase by growth hormone. Resultant transcriptional activation of ATF-2 and CHOP, cytoskeletal re-organization and mitogenesis. In: *J Biol Chem* 275 (3), S. 2103–2114.

Zinszner, H.; Kuroda, M.; Wang, X.; Batchvarova, N.; Lightfoot, R. T.; Remotti, H. *et al*. (1998): CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. In: *Genes Dev* 12 (7), S. 982–995.

7. APPENDIX

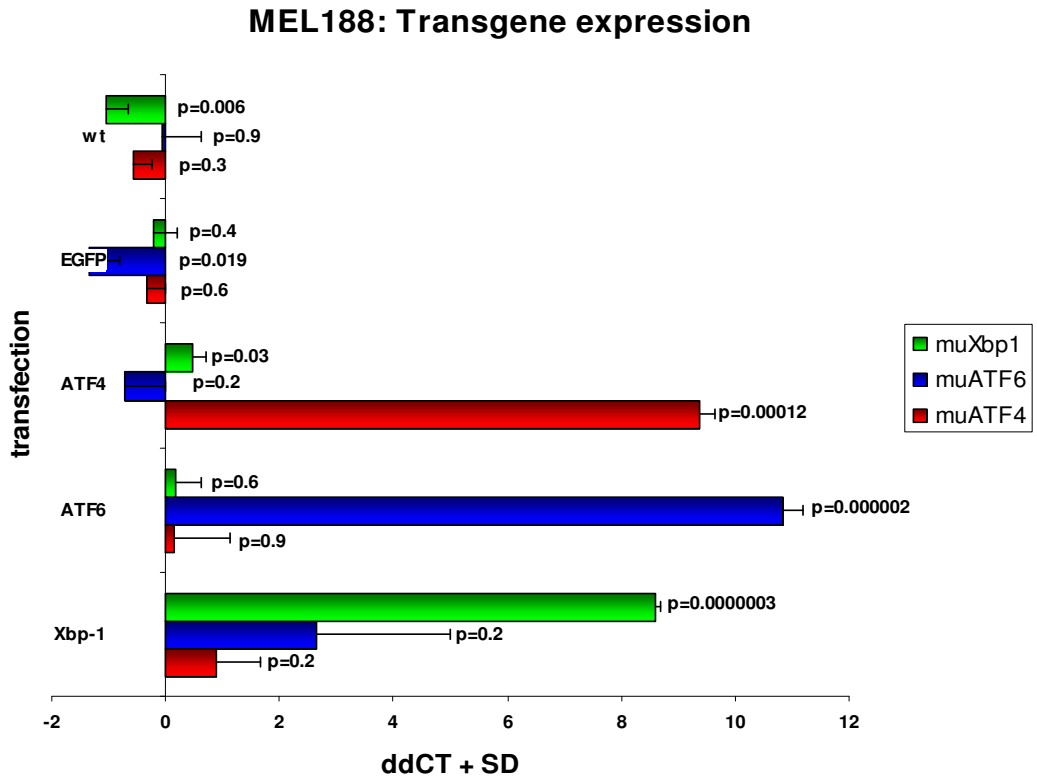
Supplementary figures and tables



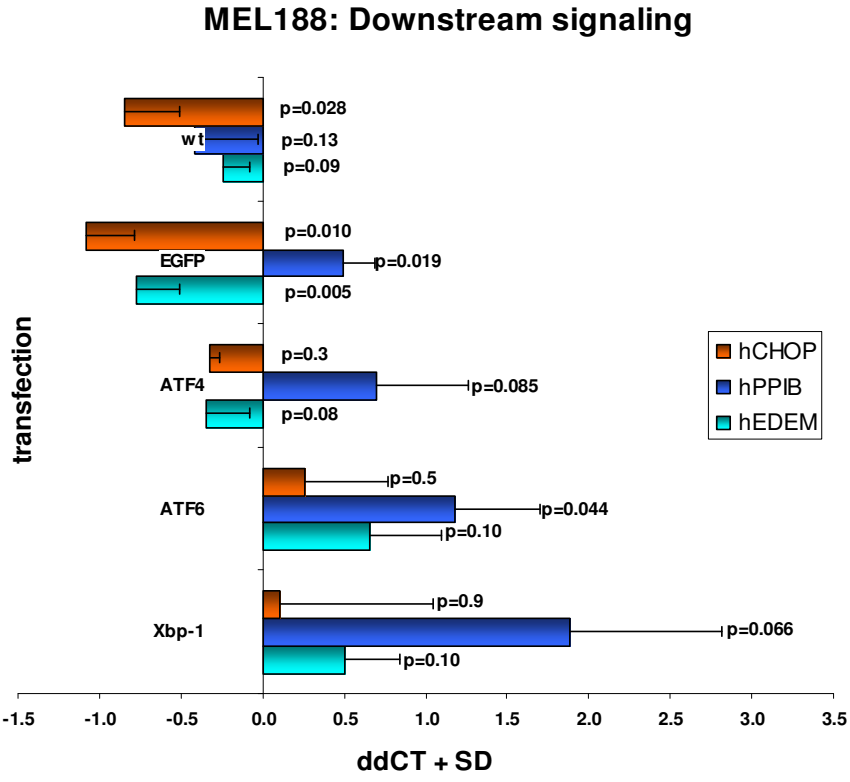
A

***Supplementary figure 1*: mRNA expression of transgenes and Chop in MLE15 and MEL188-cells (continued).**

B

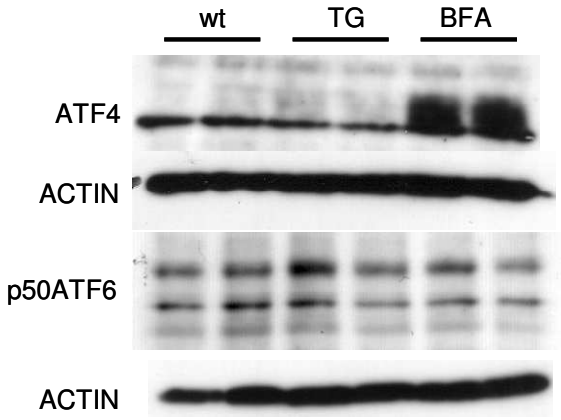


C



***Supplementary figure 1*: mRNA expression of transgenes and Chop in MLE15 and MEL188-cells.**

***Supplementary figure 1*: mRNA expression of transgenes and Chop in MLE15 and MEL188-cells.** MLE15 and MEL188 cells were transfected with Atf4, Atf6 or Xbp1 respectively. For calculation of ddCT-values, pIRES2dsRed2-transfected cells were utilized. Controls: pEGFP-N1 transfection and WT (untreated). A) Expression changes of transgenes and Chop in MLE15-cells. B) Expression of transgenes in MEL188 cells. C) Expression of Chop and additional downstream targets (Ppib and Edem) in MEL188 cells.



***Supplementary figure 2*: Protein expression of ATF4 and ATF6 after BFA and TG challenge.** ATF4 and ATF6 Protein expression of MLE12 cells treated with TG or BFA (control: WT= untreated cells) for 3 h was assessed in WB analysis.

Supplementary table 1: Results of gene set enrichment analysis (GSEA)

no.	name	no. of Genes	ATF4 22h	ATF6 22h	Xbp1 22h	ATF4 44h	ATF6 44h	Xbp1 44h	ATF4 66h	ATF6 66h	Xbp1 66h	Thapsigargin	TG 0.5h	TG 1h	TG 6h
20	Citrate cycle (TCA cycle)	29	y	y						y	y				y
51	Fructose and mannose metabolism	37								y	y	y			
52	Galactose metabolism	24								y	y				
71	Fatty acid metabolism	43								y					
100	Biosynthesis of steroids	23	y	y		y	y				y	y			
120	Bile acid biosynthesis	28								y					
190	Oxidative phosphorylation	112										y			y
230	Purine metabolism	148		y					y		y				
240	Pyrimidine metabolism	91	y	y								y			y
251	Glutamate metabolism	24		y								y			
252	Alanine and aspartate metabolism	29										y			
280	Valine, leucine and isoleucine degradation	44										y			y
290	Valine, leucine and isoleucine biosynthesis	11		y		y				y	y				
310	Lysine degradation	44		y	y										
360	Phenylalanine metabolism	20				y									
400	Phenylalanine, tyrosine and tryptophan biosynthesis	9		y											
450	Selenoamino acid metabolism	22		y											
480	Glutathione metabolism	50										y			y
510	N-Glycan biosynthesis	42		y	y	y	y	y			y	y			
511	N-Glycan degradation	14									y				
520	Nucleotide sugars metabolism	6			y										
530	Aminosugars metabolism	30										y			
532	Chondroitin sulfate biosynthesis	21		y											
533	Keratan sulfate biosynthesis	16										y			
562	Inositol phosphate metabolism	47	y	y	y					y					
563	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	22	y	y	y										
565	Ether lipid metabolism	31										y			
601	Glycosphingolipid biosynthesis - lacto and neolacto series	24									y				
604	Glycosphingolipid biosynthesis - ganglio series	15										y			
624	1- and 2-Methylnaphthalene degradation	9										y			
670	One carbon pool by folate	15										y			
680	Methane metabolism	8		y	y	y						y			
750	Vitamin B6 metabolism	5								y					
760	Nicotinate and nicotinamide metabolism	23	y												
790	Folate biosynthesis	29		y											
791	Atrazine degradation	4		y											
900	Terpenoid biosynthesis	5		y			y								
920	Sulfur metabolism	11									y				

Continue *supplementary table 1*: Results of gene set enrichment analysis (GSEA)

no.	name	no. of Genes	ATF4 22h	ATF6 22h	Xbp1 22h	ATF4 44h	ATF6 44h	Xbp1 44h	ATF4 66h	ATF6 66h	Xbp1 66h	Thapsigargin	TG 0.5h	TG 1h	TG 6h
970	Aminoacyl-tRNA biosynthesis	42		y		y						y			
980	Metabolism of xenobiotics by cytochrome P450	65										y			
982	Drug metabolism - cytochrome P450	74										y			
1030	Glycan structures - biosynthesis 1	113		y			y				y	y			
1031	Glycan structures - biosynthesis 2	59		y	y						y				
1032	Glycan structures - degradation	26				y									
1040	Biosynthesis of unsaturated fatty acids	30									y				
1100	Metabolic pathways	1070													y
3020	RNA polymerase	27		y							y				y
3022	Basal transcription factors	31		y	y					y		y			
3030	DNA replication	35		y											y
3040	Spliceosome	119													y
3060	Protein export	11		y											
3410	Base excision repair	33		y	y						y				
3420	Nucleotide excision repair	41		y							y				y
3430	Mismatch repair	22													y
3440	Homologous recombination	27		y											y
4010	MAPK signaling pathway	258	y	y	y	y			y	y	y	y			
4012	ErbB signaling pathway	86	y	y	y	y			y	y	y	y			
4070	Phosphatidylinositol signaling system	69	y	y	y					y	y				
4110	Cell cycle	111	y	y	y	y	y	y		y	y	y			y
4115	p53 signaling pathway	66	y	y	y					y		y			y
4120	Ubiquitin mediated proteolysis	131	y	y	y	y	y	y		y	y	y			
4130	SNARE interactions in vesicular transport	34	y	y	y	y	y				y				
4140	Regulation of autophagy	29	y	y	y										
4142	Lysosome	121													y
4146	Peroxisome	79													y
4150	mTOR signaling pathway	51	y	y	y	y				y	y	y			
4210	Apoptosis	85	y	y	y					y	y	y			
4310	Wnt signaling pathway	148	y	y	y							y			
4330	Notch signaling pathway	47									y				
4340	Hedgehog signaling pathway	51	y	y	y							y			
4350	TGF-beta signaling pathway	86	y	y	y							y			
4360	Axon guidance	127	y	y	y	y			y	y	y	y			
4370	VEGF signaling pathway	73	y	y	y						y	y			
4510	Focal adhesion	190	y	y	y	y		y		y	y	y			
4512	ECM-receptor interaction	77	y	y			y			y	y	y			
4520	Adherens junction	74	y	y	y					y	y	y			

Continue *supplementary table 1*: Results of gene set enrichment analysis (GSEA)

no.	name	no. of Genes	ATF4 22h	ATF6 22h	Xbp1 22h	ATF4 44h	ATF6 44h	Xbp1 44h	ATF4 66h	ATF6 66h	Xbp1 66h	Thapsigargin	TG 0.5h	TG 1h	TG 6h
4530	Tight junction	125	y	y	y					y	y	y			
4540	Gap junction	87	y	y	y	y				y	y	y			
4620	Toll-like receptor signaling pathway	94	y	y	y										
4630	Jak-STAT signaling pathway	144	y		y										
4650	Natural killer cell mediated cytotoxicity	117	y	y			y								
4660	T cell receptor signaling pathway	96	y	y	y					y		y			
4662	B cell receptor signaling pathway	67	y	y	y										
4664	Fc epsilon RI signaling pathway	78	y	y								y			
4670	Leukocyte transendothelial migration	113		y								y			
4710	Circadian rhythm	13		y											
4720	Long-term potentiation	67		y						y		y			
4740	Olfactory transduction	859													y
4810	Regulation of actin cytoskeleton	201	y	y	y	y		y		y	y	y			
4910	Insulin signaling pathway	133	y	y	y	y				y	y	y			
4912	GnRH signaling pathway	95	y	y		y						y			
4916	Melanogenesis	100										y			
4920	Adipocytokine signaling pathway	64	y	y	y					y	y				
4930	Type II diabetes mellitus	43		y	y				y						
5010	Alzheimer's disease	156		y								y			y
5012	Parkinson's disease	110										y			y
5014	Amyotrophic lateral sclerosis (ALS)	58		y											
5016	Huntington's disease	163													y
5040	Huntington's disease	31	y	y	y						y				
5050	Dentatorubropallidoluysian atrophy (DRPLA)	15	y	y	y						y				
5210	Colorectal cancer	85	y	y	y	y						y			
5211	Renal cell carcinoma	70	y	y	y	y				y	y	y			
5212	Pancreatic cancer	72	y	y	y			y		y	y	y			
5213	Endometrial cancer	52	y	y	y	y				y	y	y			
5214	Glioma	65	y	y	y	y				y	y	y			
5215	Prostate cancer	90	y	y	y	y		y		y	y	y			
5216	Thyroid cancer	27	y	y	y										
5217	Basal cell carcinoma	54		y								y			
5218	Melanoma	70	y	y						y	y	y			
5219	Bladder cancer	41	y	y	y	y				y	y	y			
5220	Chronic myeloid leukemia	75	y	y	y	y		y	y	y	y	y			
5221	Acute myeloid leukemia	56	y	y	y	y				y	y	y			
5222	Small cell lung cancer	85	y	y	y	y	y			y	y	y			
5223	Non-small cell lung cancer	54	y	y	y	y				y	y	y			
5340	Primary immunodeficiency	36			y										

Continue *supplementary table 1*: Results of gene set enrichment analysis (GSEA)

	ATF4 22h	ATF6 22h	Xbp1 22h	ATF4 44h	ATF6 44h	Xbp1 44h	ATF4 66h	ATF6 66h	Xbp1 66h	TG	TG 0.5h	TG 1h	TG 6h
Cancers	13	14	12	10	1	3	1	11	11	13	0	0	0
Signal Transduction	9	8	9	3	0	0	2	4	6	7	0	0	0
Cell Communication	4	4	4	2	0	1	0	4	4	4	0	0	0
Glycan Biosynthesis and Metabolism	1	5	3	2	2	1	0	0	5	4	0	0	0
Cell Growth and Death	3	3	3	1	1	1	0	3	2	3	0	0	2
Immune System	5	6	3	0	1	0	0	0	0	2	0	0	0
Endocrine System	3	3	2	2	0	0	0	2	2	3	0	0	0
Folding, Sorting and Degradation	2	3	2	2	2	1	0	1	2	1	0	0	0
Carbohydrate Metabolism	2	2	2	0	0	0	0	4	3	2	0	0	1
Amino Acid Metabolism	0	4	1	2	0	0	0	1	1	3	0	0	1
Replication and Repair	0	4	1	0	0	0	0	0	2	0	0	0	4
Neurodegenerative Diseases	1	3	1	0	0	0	0	0	1	2	0	0	3
Metabolism of Cofactors and Vitamins	1	3	0	0	1	0	0	1	0	2	0	0	1
Cell Motility	1	1	1	1	0	1	0	1	1	1	0	0	0
Transcription	0	2	1	0	0	0	0	1	1	1	0	0	2
Development	1	1	1	0	0	0	1	1	1	1	0	0	0
Nucleotide Metabolism	1	2	0	0	0	0	1	0	1	1	0	0	1
Energy Metabolism	0	1	1	1	0	0	0	0	1	2	0	0	1
Signaling Molecules and Interaction	1	1	0	0	1	0	0	1	1	1	0	0	0
Transport and Catabolism	1	1	1	0	0	0	0	0	0	0	0	0	2
Lipid Metabolism	0	0	0	0	0	0	0	2	1	1	0	0	0
Metabolic Diseases	0	1	1	0	0	0	1	0	0	0	0	0	0
Translation	0	1	0	1	0	0	0	0	0	1	0	0	0
Nervous System	0	1	0	0	0	0	0	1	0	1	0	0	0
Metabolism of Other Amino Acids	0	1	0	0	0	0	0	0	0	1	0	0	1
Xenobiotics Biodegradation and Metabolism	0	1	0	0	0	0	0	0	0	2	0	0	0
Metabolism of Terpenoids and Polyketides	0	1	0	0	1	0	0	0	0	0	0	0	0
Immune System Diseases	0	0	1	0	0	0	0	0	0	0	0	0	0
Environmental Adaptation	0	1	0	0	0	0	0	0	0	0	0	0	0
Metabolism	0	0	0	0	0	0	0	0	0	0	0	0	1
Sensory System	0	0	0	0	0	0	0	0	0	0	0	0	1

Continue *supplementary table 1*: Results of gene set enrichment analysis (GSEA)

The first table (three pages) lists the KEGG-pathways significantly regulated (adj. p <0.05) in at least one of the contrasts from the arrays. A “y” labels the individual contrasts in which the pathway was regulated. TG 0.5 h, TG 1 h and TG 6 h stand for the contrasts of TG-treated versus WT- MLE12-cells. The pathway analysis for those contrasts was performed separately. The other contrasts are pathways in the respective contrast belong to the indicated categories.

***Supplementary table 2*: Development of body weight in transgenic animals**

Body weight

line	genotype	time of induction	induced change in weight [g]	±	n	control change in weight [g]	±	n
ATF4	+-/+-	4 weeks	3.9	1.0	5	3.0	0.7	5
ATF4	+-/+-	16 weeks	7.7	1.1	4	5.5	1.8	5
ATF4	+-/+-	32 weeks	7.2	2.0	5	7.0	1.3	5
ATF4	++/++	4 weeks	3.2	1.0	5	2.3	1.2	5
ATF4	++/++	32 weeks	6.7	1.9	5	8.2	0.8	4
ATF6	+-/+-	4 weeks	1.8	0.5	5	2.5	0.3	5
ATF6	+-/+-	16 weeks	5.8	1.9	4	6.4	0.3	5
ATF6	+-/+-	32 weeks	6.0	2.7	5	5.4	1.7	4
ATF6	++/++	4 weeks	6.7	3.3	5	5.7	3.1	5
Xbp1	+-/+-	4 weeks	2.7	1.3	5	2.9	0.6	5
Xbp1	+-/+-	8 weeks	4.9	1.0	3	5.6	0.9	2
Xbp1	+-/+-	32 weeks	6.7	1.4	5	5.9	1.5	5

The table lists the changes in body weight of the mice in the experiment from beginning of the experiment to its finalization after the indicated time of induction ± the standard deviation, separately for controls and Dox induced animals and the number of animals in each group (n). Significant differences (p<0.05) are highlighted in blue.

***Supplementary table 3*: Cells countings and cellular composition in the broncho-alveolar lavage (BAL)**

Cells in the BAL

			induced			control				
line	genotype	time of induction	cells in lavage [cells/ml]	±	n	cells in lavage [cells/ml]	±	n		
ATF4	+-/+-	4 weeks	N/A	-	-	N/A	-	-		
ATF4	+-/+-	16 weeks	267500	130815	2	170000	50166	3		
ATF4	+-/+-	32 weeks	N/A	-	-	N/A	-	-		
ATF4	++/++	4 weeks	222000	89920	5	429900	329866	5		
ATF4	++/++	32 weeks	57500	20917	5	60625	11968	4		
ATF6	+-/+-	4 weeks	N/A	-	-	N/A	-	-		
ATF6	+-/+-	16 weeks	65000	4564	4	112500	36954	5		
ATF6	+-/+-	32 weeks	96500	48173	5	98750	11792	4		
ATF6	++/++	4 weeks	75500	15949	5	95000	22430	5		
Xbp1	+-/+-	4 weeks	N/A	-	-	N/A	-	-		
Xbp1	+-/+-	8 weeks	N/A	-	-	N/A	-	-		
Xbp1	+-/+-	32 weeks	110500	48907	5	106000	52040	5		

Cell differentiation

			induced		control		induced		control		induced		control		induced	control
line	genotype	time of induction	macrophages [%]	±	macrophages [%]	±	lymphocytes [%]	±	lymphocytes [%]	±	PMN [%]	±	PMN [%]	±	n	n
ATF4	+-/+-	4 weeks	95.2	7.4	99.2	0.3	3.8	5.7	0.5	0.0	1.0	1.7	0.3	0.3	3	3
ATF4	+-/+-	16 weeks	82.5	10.6	89.0	15.6	1.0	0.0	0.3	0.4	16.5	10.6	10.8	15.2	2	2
ATF4	+-/+-	32 weeks	89.8	8.5	95.0	2.8	2.9	3.1	3.8	2.6	7.3	6.6	1.1	0.8	5	5
ATF4	++/++	4 weeks	99.4	0.9	99.7	0.7	0.3	0.4	0.0	0.0	0.3	0.7	0.3	0.7	5	5
ATF4	++/++	32 weeks	92.3	10.4	89.6	19.8	7.5	10.5	9.4	18.8	0.2	0.4	1.0	1.1	5	4
ATF6	+-/+-	4 weeks	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	-	-
ATF6	+-/+-	16 weeks	92.5	12.4	83.9	14.8	1.5	1.7	1.9	1.2	6.0	10.7	14.2	13.8	4	5
ATF6	+-/+-	32 weeks	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	-	-
ATF6	++/++	4 weeks	99.0	2.2	99.6	0.2	0.2	0.4	0.3	0.3	0.8	1.8	0.1	0.2	5	5
Xbp1	+-/+-	4 weeks	97.7	3.2	98.3	1.2	0.7	0.6	1.0	0.0	1.7	2.9	0.7	1.2	3	3
Xbp1	+-/+-	8 weeks	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	-	-
Xbp1	+-/+-	32 weeks	89.2	13.2	91.3	9.6	2.0	2.5	3.2	3.4	8.8	10.7	5.5	6.4	5	5

Continue *supplementary table 3*: Cells countings and cellular composition in the broncho-alveolar lavage (BAL)

The tables list the cell numbers in the BAL (upper table) and the result of the differentiation (lower table) of BAL-cells body weight of the mice at the end of the indicated experimental times of induction, ± the standard deviation, separately for controls and Dox induced animals and the number of animals in each group (n). Significant differences ($p<0.05$) are highlighted in blue.

***Supplementary table 4*: Animal deaths**

Deaths

line	genotype	time of induction	induced deaths	control deaths
ATF4	+-/+-	4 weeks	0	0
ATF4	+-/+-	16 weeks	1	0
ATF4	+-/+-	32 weeks	0	0
ATF4	++/++	4 weeks	0	0
ATF4	++/++	32 weeks	0	1
ATF6	+-/+-	4 weeks	0	0
ATF6	+-/+-	16 weeks	1	0
ATF6	+-/+-	32 weeks	0	1
ATF6	++/++	4 weeks	0	0
Xbp1	+-/+-	4 weeks	0	0
Xbp1	+-/+-	8 weeks	0	0
Xbp1	+-/+-	32 weeks	0	0

The table lists the number of animal deaths, separately for each experimental group, during the indicated period of transgene induction.

***Supplementary table 5*: Effects of transgene induction on lung compliance**

Compliance

			induced			control		
line	genotype	time of induction	compliance [ml/(kPa*Kg)]	±	n	compliance [ml/(kPa*Kg)]	±	n
ATF4	+-/+-	4 weeks	5.7	1.1	3	5.8	2.1	3
ATF4	+-/+-	16 weeks	7.4	1.7	2	8.0	0.6	3
ATF4	+-/+-	32 weeks	8.7	1.7	5	8.4	1.1	5
ATF4	++/++	4 weeks	6.6	0.9	5	6.2	0.7	5
ATF4	++/++	32 weeks	7.1	1.0	5	8.6	1.3	4
ATF6	+-/+-	4 weeks	N/A	-	-	N/A	-	-
ATF6	+-/+-	16 weeks	7.7	1.4	4	7.6	1.2	5
ATF6	+-/+-	32 weeks	N/A	-	-	N/A	-	-
ATF6	++/++	4 weeks	N/A	-	-	N/A	-	-
Xbp1	+-/+-	4 weeks	6.3	1.6	3	7.3	0.9	3
Xbp1	+-/+-	8 weeks	N/A	-	-	N/A	-	-
Xbp1	+-/+-	32 weeks	8.1	2.1	5	9.7	1.9	5

The average lung compliance ± standard deviation and the n-numbers are listed separately for induced and non-induced mice from each experimental group. There are no significant differences between the induced and non-induced groups.

Supplementary table 6: Effects of transgene induction on running time in treadmill-test

Treadmill

line	genotype	time of induction	induced change running time [min]	±	n	control change running time [min]	±	n
ATF4	+-/+-	4 weeks	N/A	-	5	N/A	-	5
ATF4	+-/+-	16 weeks	N/A	-	4	N/A	-	5
ATF4	+-/+-	32 weeks	N/A	-	5	N/A	-	5
ATF4	++/++	4 weeks	6.1	2.8	5	6.4	2.6	5
ATF4	++/++	32 weeks	7.6	6.6	5	0.7	4.9	4
ATF6	+-/+-	4 weeks	N/A	-	5	N/A	-	5
ATF6	+-/+-	16 weeks	5.7	1.6	4	1.9	3.7	5
ATF6	+-/+-	32 weeks	3.5	8.0	5	2.7	5.1	4
ATF6	++/++	4 weeks	3.4	4.8	5	7.2	3.9	5
Xbp1	+-/+-	4 weeks	N/A	-	5	N/A	-	5
Xbp1	+-/+-	8 weeks	N/A	-	3	N/A	-	2
Xbp1	+-/+-	32 weeks	7.6	5.5	5	1.9	2.9	5

The average running time ± standard deviation and the n-numbers are listed separately for induced and non-induced mice from each experimental group. There are no significant differences between the induced and non-induced groups.

***Supplementary table 7*: Genes of interest and their regulation by ATF4, ATF6, XBP1 and TG**

Regulation by	ATF4	ATF6	XBP1	ATF4	ATF6	XBP1	ATF4	ATF6	XBP1	TG	TG		
treatment / transfection time	22h			44h			66h			22h / 44h	0.5h	1h	6h
Genename													
Acta2	-	-	-	-	-	-	-	-	-	-0.61	-	-	-0.88
ATF2	-	-	-	-	-	-	-	-	-	0.51	-	-	-0.61
ATF3	-	-0.98	-0.65	-	-0.58	-	-	-	-	1.29	-	1.29	1.97
Atf4	3.38	0.48	-	1.47	-	-	-	-	-	2.07	0.56	1.26	1.57
ATF5	0.64									2.09	-	-	1.19
Atg12	-	-	0.47	-	-	-	-	-	-	0.37	-	0.45	1.11
Bcl2	-	0.72	-	-	-	-	-	-	-	-0.36	-	-	-0.46
Cav1	-	-	-	-	-	-	-	-	-	-0.66	-	-	-1.28
Cdh1	-	-	-	-	-	-	-	-	-	-1.35	-	-	-1.14
Ddit3	-	1.12	-	-	-	-	-	-	-	3.52	-	1.42	2.91
Dnajc10	-	0.37	-	-	0.52	-	-	-	-	-	-	-	-0.55
Edn1	-	-0.88	-	-	-	-	-	-	-	-	-	-	-
Eif2ak2	-	-0.66	-	-	-	-	-	-0.65	-	-1.02	-	-	-
Ep300	-0.73	-0.75	-0.75	-	-	-	-	-	-	-	-	-	-
Fbxw11	-	-	-	-	-	-	-	-	-	-	-	-	-
Gja1	-	-	-	-	-	-	-	-	-	-	-	-	-1.78
Gsn	-	-	-	-	-	-	-	-	-	-1.27	-	-	-1.26
Gss	-	-	-	-	-	-	-	-	-	-	-	-	-
Hmox1	-	-0.39	-	-	-	-	-	-	-	1.62	-	-	-
Il1a	-	-	-	-	-	-	-	-	-	-	-	-	-
Il1b	-	-	-	-	-	-	-	-	-	-	-	-	-
Map3k5	-	-0.35	-	-	-	-	-	-	-	-0.25	-	-0.36	-0.52
Mapk14	-	-0.24	-	-	-	-	-	-	-	-0.30	-	-	0.44
Myd116	-	-0.91	-	-	-	-	-	-	-	2.40	-	-	2.39
Nfe2l2	-	-	-	-	-	-	-	-	-	-0.35	-	-	-1.34
Nfkbia	-	-	-	-	-	-	-	-	-	-0.91	-	1.04	1.13
Nfkbie	-	-0.50	-	-	-	-	-	-	-	-0.34	-	-	1.79
nfya	-	-	-	-	-	-	-	-	-	0.41	-	-	-
nfyb	-	-	-	-	-	-	-	-	-	-	-	-	0.44
nfyc	-	-0.57	-	-	-	-	-	-	-	-	-	-	0.58
Nov	-	-	-	-	-	-	-	-	-	-0.31	-	-	-0.34
Pcaf	-	-	-	-	-	-	-	-	-	-0.40	-	-	-0.62
Pdgfa	-	-0.34	-	-	-	-	-	-	0.54	-1.03	-	-	-
Pdgfb	-	-0.49	-	-	-	-	-	-	-	-0.83	-	-	-
Plat	-	-0.34	-	-	-	-	-	-	-	-1.40	-	-	-1.42
Plau	-	-	-	-	-	-	-	-	-	-0.44	-	-	-
Ppp1r15b	-	-0.35	-	-	-	-	-	-	-	-0.42	-	-	-0.30
Ptges2	-	-0.70	-	-	-	-	-	-	-	-0.53	-	-	0.51
Rela	-	-0.91	-0.81	-	-	-	-	-	-	-	-	-	1.25
Runx3	-0.41	-0.35	-	-	-	-	-	-	-	-	-	-	-
Serpine1	-	-	-	-	-0.59	-	-	-	-	-1.22	-	0.65	-1.14
Sirt1		-0.55	-0.69	-	-	-	-	-	-	0.62	-	-	1.91
Sod3	-	-	-	-	-	-	-	-	-	-	-	-	-
Stch	-	0.55	1.16	-	-	-	-	-	-	0.69	-	-	-
Tgfb1	-	-0.74	-	-	-	-	-	-	-	-0.86	-	-	-
Tgfb2	-	-	-	-	-	-	-	-	-	-	-	-	-0.98
Tgfb3	-	-	-	-	-	-	-	-	-	-1.64	-	-	-1.08
Tjp1	-	-0.49	-	-	-	-	-	-	-	-	-	-	-
Tlr2	0.73	-0.64	-	-	-	-	-	-	-	-	-	0.54	-
Tnf	-	-	-	-	-	-	-	-	-	-	-	-	-
Trib3	1.05	0.50	-	-	-	-	-	-	-	3.10	-	-	3.69
Txlnb	-	-	-	-	-	-	-	-	-	-	-	-	-
Upf1	-	-0.73	-	-	-	-	-	-	-	-0.53	-	-	0.78
Wfs1	-	1.68	1.31	-	-	0.55	-	-	-	1.41	-	-	-
Xbp1		1.84	5.34		1.58	3.87			1.78	1.77		0.75	2.04
Yy1	-	0.59	-	-	-	-	-	-	-	-0.38	-	-0.75	-

The table lists the regulation of selected genes in microarrays in lfc. "-" = no significant regulation. When a gene was represented by more than one probe, the strongest regulation is depicted.

Acknowledgements

First, I would like to say thank you, to all the people who contributed to this Thesis with practical, experimental work. Their individual contributions are generally mentioned in the text and shall not be repeated here. Nevertheless I want to express my special gratitude to Ingrid Henneke, for her work with the animals and Jochen Wilhelm for his contributions to the microarray experiments.
During my research, the graduate programmes “Molecular Biology and Medicine of the Lung” (MBML), the “Giessen Graduate School for the Life sciences” GGL and the “international PhD-programme of the faculties of veterinary medicine and medicine” were greatly accompanying and contributing to my scientific education, for which I am very grateful.
I thank Clemens Ruppert for writing the proposal for the animal experiments.
I want to thank all colleagues who helped me with advice and encouragement. In this context, it is my special wish to express my gratitude to Martina Korfei, also for numerous scientific discussions until late at night.
Prof. Tim Weaver (Cincinnati) was kindly accompanying my work from the beginning, without being officially involved. Therefore he earned my special thanks. I thank Julia Grabitzki and Dorothea Hühn for critically reading my manuscript.
I want to extend my thanks to Prof. Renkawitz, who agreed to take the responsibility to be co-supervisor to my work. Last but not least I want to thank my supervisor Prof. Andreas Günther. I feel honoured that I was entrusted with a project of such (also financial) dimensions at the first place. The more thankful I am to him, for personally taking care and showing special interest in my project.

Publications

Paper

Jaé, N., Wang, P., Gu, T. Hühn, M., Palfi, Z., Urlaub, H., Bindereif, A. Essential Role of a Trypanosome U4-Specific Sm Core Protein in Small Nuclear Ribonucleoprotein Assembly and Splicing. EUKARYOTIC CELL (2010) 9(3):379–386

Oral presentations

Hühn M., Korfei M., Ruppert C., Lohfink D., Seeger W., Günther A. Signalpfade und zelluläre Konsequenzen der Überexpression von ER-Stress Sensormolekülen in vitro und in vivo. 51.Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V., Hannover, Germany, 17-20 March, 2010

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Henneke, I., Seeger, W., Günther, A. ER-stress signalling in the alveolar epithelium. 3rd Conference of the Giessen graduate school for the Life Sciences (GGL), Giessen, Germany, 29-30 September, 2010

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Henneke, I., Seeger, W., Günther, A. Differential gene regulation in the murine alveolar epithelium *in vitro* and *in vivo* by over-expression of the ER-stress sensor molecules ATF4, ATF6α (N-terminal Domain) and XBP1 (spliced). ECCPS Area D workshop, Frankfurt am Main, Germany, September 12, 2008

Hühn, M. Differential gene regulation in the murine alveolar epithelium *in vitro* and *in vivo* by over-expression of the ER-stress sensor molecules ATF4, ATF6α (N-terminal Domain) and XBP1 (spliced) MBML annual retreat, Rauischholzhausen, Germany, July 28-30, 2008

Hühn, M. Over-expression of ER-stress signalling molecules in the murine alveolar epithelium in vitro and in vivo. MBML annual retreat. Rauischholzhausen, Germany, July 27-29, 2009

Hühn, M., ER-stress signalling in the alveolar epithelium MBML annual retreat, Rauischholzhausen, Germany, August 9-11, 2010

Poster presentations

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Henneke, I., Seeger, W., Günther, A. ER-stress signalling in the alveolar epithelium. The European IPF Network – translational approach to a disastrous disease Athens, Greece, November 14th, 2010

Hühn, M., Korfei, M., Ruppert, C., Lohfink D., Henneke, I., Seeger, W., Günther, A. Over-expression of ER-stress sensor molecules in the murine alveolar epithelium in vitro and in vivo. Annual Meeting of the German and British Societies for Matrix Biology, Frankfurt am Main, Germany, March 18th-20th, 2010

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Seeger, W., Günther, A. Signalling pathways and cellular consequences in response to over-expression of the ER-stress sensor molecules ATF4, ATF6α (N-terminal Domain) and spliced XBP1 in the murine alveolar epithelium *in vitro* and *in vivo. 1st* Conference of the Giessen graduate school for the Life Sciences (GGL), Giessen, Germany, September 30- October 1, 2008

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Seeger, W., Günther, A. Signalling pathways and cellular consequences in response to over-expression of ER-stress signalling molecules in the murine alveolar epithelium in vitro and in vivo. 2nd Conference of the Giessen graduate school for the Life Sciences (GGL), Giessen, Germany, September 30th – October 1st, 2009

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Seeger, W., Günther, A. Signalpfade und zelluläre Konsequenzen der Überexpression von ER-Stress Sensormolekülen *in vitro* und *in vivo.* Herbsttreffen der Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V. Freiburg. November 7-8, 2008

Hühn, M., Korfei, M., Ruppert, C., Lohfink, D., Seeger, W., Günther, A. Signalling pathways and cellular consequences in response to over-expression of the ER-stress sensor molecules ATF4, ATF6α (N-terminal Domain) and spliced XBP1 in the murine alveolar epithelium in vitro and in vivo. ECCPS retreat. Max-Planck-Institute Bad Nauheim, Germany, July 3-4, 2009

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