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Studies on the stress-inducible pseudokinase TRB3, a novel inhibitor of transcription factor ATF4

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which will be referred to in the text by their Roman numerals:

I **Örd, D**. and Örd, T. (2003) Mouse NIPK interacts with ATF4 and affects its transcriptional activity. *Exp. Cell Res.*, 286, 308–320.

II **Örd, D**. and Örd, T. (2005) Characterization of human NIPK (TRB3, SKIP3) gene activation in stressful conditions. *Biochem. Biophys. Res. Commun.*, 330, 210–218.

III **Örd, D**., Meerits, K. and Örd, T. (2007) TRB3 protects cells against the growth inhibitory and cytotoxic effect of ATF4. *Exp. Cell Res.*, 313, 3556–3567.

LIST OF ABBREVIATIONS

AARE	—	amino acid response element
Akt/PKB	—	protein kinase B
AML	—	acute myeloid leukemia
Asns	—	asparagine synthetase
ATF	—	activating transcription factor
BMP	—	bone morphogenetic protein
bp	—	base pair(s)
bZip	—	basic region-leucine zipper
C-	—	carboxy-(terminus of the protein)
C/EBP	—	CCAAT/enhancer binding protein
CHOP	—	C/EBP homologous protein
CRE	—	cyclic AMP responsive element
eIF2α	—	eukaryotic translation initiation factor 2α
EMSA	—	electrophoretic mobility shift assay
ER	—	endoplasmic reticulum
ERK	—	extracellular signal regulated protein kinase
GADD34	—	growth arrest- and DNA damage-inducible gene 34
grp78	—	glucose-regulated protein 78
hTRB3	—	human TRB3
IRE1	—	inositol-requiring enzyme 1
ISR	—	integrated stress response
JNK	—	cJun N-terminal kinase
kb	—	kilobasepairs
m	—	mouse (protein)
MAPK	—	mitogen-activated protein kinase
MKK	—	mitogen-activated protein kinase kinase
N-	—	amino-(terminus of the protein)
NF-κB	—	nuclear factor-κB
Nrf2	—	NF-E2 related factor 2
NSRE-1	—	nutrient stress response element-1
PERK	—	PKR-like endoplasmic reticulum kinase
PKR	—	protein kinase RNA-dependent
RNAi	—	RNA interference
ROS	—	reactive oxygen species
siRNA	—	small interfering RNA
tet	—	tetracycline
TRB	—	Tribbles homolog
uORF	—	upstream open reading frame
UPR	—	unfolded protein response
UTR	—	untranslated region

1. INTRODUCTION

The cellular response to environmental changes is mediated by a diverse array of intracellular signaling pathways that relay and integrate the signals, resulting in either growth, proliferation, differentiation or apoptosis. When environmental changes perturb the cellular homeostasis, the stress response is generated by the stress-activated signal transduction pathways that direct the changes in gene expression to ensure adaption to stress (that is essential for a long-term cell survival) or, if adaption fails, the initiation of the cell death program.

Activating transcription factor 4 (ATF4), a basic region-leucine zipper transcription factor, plays a crucial role in managing the integrated stress response (ISR) evoked by a variety of adverse conditions, including metabolic, oxidative, and endoplasmic reticulum stresses. In ISR, ATF4 regulates the expression of the genes involved in protein biosynthesis and in the control of ATF4 pathway activation and the programmed cell death. The activity of ATF4 must be strictly regulated, because both the deficiency of ATF4 and its excessive activity sensitize a cell to death. The altered activity of the ATF4 pathway has been implicated in the pathogenesis of several illnesses, including diabetes, ischemia and neurodegenerative diseases. The control mechanisms of the ATF4 transcriptional activity include inhibitory proteins and negative feedback loops that attenuate the stress signaling. One such regulator of ATF4 is pseudokinase Tribbles homolog 3 (TRB3). *TRB3* was identified in our lab as a stress-responsive gene markedly upregulated in neuronal GT1-7 cells in response to the disruption of calcium homeostasis. My studies in the lab and the resulting publications that form the basis of the current thesis have dealt mainly with the stress-dependent activation of *TRB3* and its role in the stress response. These studies have helped to establish *TRB3* as an ATF4 target gene and contributed to the elucidation of a novel feedback regulatory mechanism for ATF4.

2. REVIEW OF LITERATURE

2.1. The structure of the Tribbles family proteins

The *D. melanogaster* protein Tribbles is the prototype representative of the novel protein family which also includes three distinct mammalian proteins, Tribbles homolog 1, 2 and 3 (TRB1, TRB2 and TRB3). The members of this family are featured by a serine/threonine (S/T) protein kinase domain, which contains critical substitutions in the amino acid sequence compared to the canonical S/T kinase sequence. The protein kinase domain consists of approximately 250–300 amino acids and contains 12 conserved subdomains, the first four of which are mainly responsible for the ATP binding, while the last seven subdomains (VIA-XI) are primarily involved in the binding of the peptide substrate (Hanks and Hunter, 1995). Within the kinase domain, three peptide motifs are thought to be critical for a catalytic function. Each of these motifs contains a highly conserved amino acid residue that participates in phosphotransfer: first, the invariant Lys in motif Val-Ala-Ile-Lys (subdomain II) participates in anchoring and orienting the ATP; second, the catalytic Asp in motif His-Arg-Asp (subdomain VIB) acts as a proton acceptor; and third, the invariant Asp in highly conserved motif Asp-Phe-Gly (subdomain VII) chelates Mg^{2+} ions to orient the γ-phosphate of ATP for the transfer (Manning *et al.*, 2002).

The alignment of amino acid sequences of the Tribbles family proteins (Fig. 1) reveals that the proteins are well conserved in the region corresponding to subdomains VIA to XI, but significantly less similarity is observed in the N-terminal part of the proteins. The comparison of the kinase domain sequences amongst the Tribbles family proteins reveals that human (h)TRB3 shares 55% identity to hTRB2, and 54% identity to hTRB1, whereas the degree of identity between the kinase domains of hTRB3 and Tribbles is considerably less (45%). The highest sequence identity to the kinase domain of TRB proteins possesses Ca^{2+}/calmodulin-dependent kinases from the SNF1/AMPK family. MELK, the enzymatically active member of SNF1/AMPK family (Gil *et al.*, 1997), has about 30% identity to the kinase domain of TRB3. However, the TRB proteins have several changes in the peptide motifs that are catalytically crucial. As seen in Fig. 1, TRB proteins contain the Lys that is crucial for the ATP binding in subdomain II, but they lack the classical glycines-containing region in subdomain I, which collaborates in anchoring the ATP (Hegedus *et al.*, 2007). Further, the catalytic Asp is conserved in the subdomain VIB of TRB proteins, but the nearly invariant His of catalytic core is not conserved. The most striking difference from the conventional kinases is the lack of DFG triplet in subdomain VII, which strongly predicts that TRB proteins have no kinase activity (Manning *et al.*, 2002). It is relevant to mention that MELK with the abrogated DFG motif (MELK-D150A) is a

Figure 1. Comparison of the amino acid sequences of mouse TRB3 (mTRB3; the NCBI protein database accession number CAD55728), human TRB3 (hTRB3; CAG27047), human TRB2 (hTRB2; AAO89231), human TRB1 (hTRB1; AAK58174), *Drosophila* Tribbles (AAF26374), and human SNF1/AMPK family kinase MELK (hMELK; NP_055606) (only residues 1–300 are shown). The alignment is performed by the MUSCLE software (http:www.ebi.ac.uk/muscle); residues on solid and gray background are identical and similar, respectively, to the column consensus. The kinase(-like) domain is defined by ScanProsite software (ExPASy). The protein kinase subdomains II and V-XI are indicated (Hanks and Hunter, 1995). The peptide motifs that are critical for catalytic function are underlined and the highly concerved residues of these motifs are marked by an asterisk. Percentage identity of sequences is calculated for kinase(-like) domains only.

kinase-dead mutant (Vulsteke *et al.*, 2004). To date, neither autophosphorylation nor phosphorylation of another protein by TRB proteins has been experimentally detected (Wilkin *et al.*, 1997; Grosshans and Wieschaus, 2000; Bowers *et al.*, 2003). Therefore, the Tribbles family proteins are presently classified as pseudokinases or kinase-like proteins (Boudeau *et al.*, 2006).

The kinase-like domain of TRB proteins is flanked by relatively short N- and C-terminal segments. The amino acid composition of the terminal segments is less conserved within the Tribbles family, nevertheless they have some common features. For example, the N-terminal segment has a very high serine and proline content that may cause the instability of protein (Hegedus *et al.*, 2007). In addition, the N-terminal domain of hTRB1 and hTRB3, but not hTRB2, contains a nucler localization signal (Kiss-Toth *et al.*, 2005). The C-terminal motif [DE]-Q-x-V-P-[DE] of TRB proteins is recognized by E3 ubiquitin ligase COP1 (constitutive photomorphogenic protein 1) and may be involved in the TRB-dependent degradation of the target proteins (Qi *et al.*, 2006). The TRB family pseudokinases regulate various physiological and developmental processes by the mechanism involving direct protein-protein interactions, as will be described below.

2.2. Tribbles acts as a blocker of mitosis

In the year 2000, *Tribbles* was identified simultaneously in three independent developmental screens of *Drosophila* as a gene that is required for the block of the mitosis of invaginating mesodermal precursor cells during gastrulation (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000). Further analysis revealed that Tribbles promotes the proteasomal degradation of protein phosphatase Cdc25/String and thereby delays mitosis. While Tribbles functions in the same way to regulate the mitosis of germ line cells during *Drosophila* oogenesis and imaginal disc cells during wing formation, it does not act as a general mitosis inhibitor (Mata *et al.*, 2000; Seher and Leptin, 2000). Tribbles is also a negative regulator of C/EBP family transcription factor Slbo, the optimal expression level and the activity of which is critical for border cell migration during *Drosophila* oogenesis. By binding to Slbo, Tribbles controls the level of Slbo protein *via* stimulating Slbo ubiquitination and proteolysis (Rorth *et al.*, 2000). The additional experiments by Rorth *et al*. (2000) showed that Tribbles does not stimulate protein turnover in a general fashion but only affects specific targets in a context-dependent manner.

Taken together, the data indicate that Tribbles regulates the embryonic development of *Drosophila* at several stages by downregulating Cdc25/String and Slbo protein expression through promoting their proteolytic degradation. The discovery of the biological role of Tribbles has prompted the researchers to investigate the possible involvement of mammalian homologs of Tribbles in the control of the cell cycle and proteasomal degradation.

2.3. The functional roles of TRB1 and TRB2

The human ***Tribbles homolog 1*** (*TRB1*, *Trib1*, *c8FW*) gene is located on chromosome 8 at q24.13. The TRB1 protein has been reported to regulate the mitogen activated protein kinase (MAPK) pathway by binding directly to MAPK kinases MEK-1 and MKK4, leading to the enhanced phosphorylation and activity of the extracellular signal regulated protein kinase (ERK) (Kiss-Toth *et al.*, 2004). As recently described, TRB1 can be involved in the pathogenesis of atherosclerosis *via* the regulation of the MAPK signaling pathway (Sung *et al.*, 2007). According to this report, TRB1 was found to be selectively overexpressed in chronically inflamed human atherosclerotic arteries, where it associates with MKK4 and controls the cJun N-terminal kinase (JNK) activity and thereby the proliferation and chemotaxis of vascular smooth muscle cells.

TRB1 has also been implicated in the modulation of the antimicrobial response in macrophages by regulating CCAAT/enhancer binding protein β (C/EBPβ)-dependent gene expression (Yamamoto *et al.*, 2007b). This hypothesis is supported by the results, which demonstrate that in lipopolysaccharide-stimulated macrophages, TRB1-deficiency augments the level of C/EBPβ protein and C/EBPβ-dependent gene transcription, while the overexpressed TRB1 binds to C/EBPβ and downregulates its level resulting in the inhibition of C/EBPβ-dependent gene expression.

By several other studies, TRB1 is considered to be a novel myeloid oncogene that participates in the pathogenesis of acute myeloid leukemia (AML). *TRB1* gene was found to be upregulated in AML cases carrying double minutes – circular, extra-chromosomal amplifications of the segment from 8q24 that always include the *MYC* gene, a major oncogene (Storlazzi *et al.*, 2004; Storlazzi *et al.*, 2006; Rothlisberger *et al.*, 2007). Recently Jin *et al*. (2007) reported that ectopically expressed TRB1 is able to transform the primary bone marrow cells and significantly accelerate the development of AML that has been induced by other myeloid oncogenes, like homeobox genes *Hoxa9* and *Meis1*. The proposed mechanism for TRB1 transforming activity involves the enhancing of ERK phosphorylation which protects leukemic cells against apoptosis (Jin *et al.*, 2007). TRB1 has also been shown to bind to the arachidonic acid metabolizing enzyme 12-lipoxygenase (12-LOX), which is overexpressed in a variety of human tumors, and plays a role in tumor progression and metastasis (Tang *et al.*, 2000).

The human ***Tribbles homolog 2*** (*TRB2*, *Trib2*, *c5FW*) gene is located on chromosome 2 at p24.3. TRB2 was first characterized as a labile cytoplasmic phosphoprotein whose mRNA is upregulated by mitogens in dog thyroid cells (Wilkin *et al.*, 1997).

Similarly to TRB1, TRB2 has also been identified as a novel myeloid oncogene involved in the pathogenesis of AML. TRB2 is suggested to be

oncogenic due to the ability to inactivate the transcription factor C/EBPα that is a critical factor for myeloid development and is frequently deregulated in AML (Keeshan *et al.*, 2006). According to the study, *TRB2* expression was preferentially elevated in a subset of human AML patients exhibiting C/EBPα defects. At the same time, exogenously expressed TRB2 can inactivate C/EBPα *via* binding to it and promoting the proteolytic degradation of C/EBPα.

In addition to its role in cancer, TRB2 has been implicated in another pathological process. Namely, TRB2 has been identified as an autoantigen in autoimmune uveitis, an ocular inflammatory disorder that can lead to vision loss (Zhang *et al.*, 2005).

Opposite to the anti-apoptotic role of TRB2 in AML reported by Keeshan *et al*. (2006), the upregulation of TRB2 in response to the survival factor withdrawal is considered to be pro-apoptotic in hematopoietic cells, leading to the mitochondrial dysfunction and degradation of the prosurvival protein Mcl-1 (Lin *et al.*, 2007). Consistent with this finding, the knockdown of TRB2 suppresses apoptosis induced by the granulocyte macrophage colony-stimulating factor deprivation.

The involvement of TRB2 in the differentiation of adipocyte has been studied by Naiki *et al*. (2007) demonstrating that TRB2, but not TRB1, is downregulated immediately upon the induction of 3T3-L1 preadipocytes to differentiate. Further biochemical studies revealed that TRB2 inhibits the adipocyte differentiation through two distinct mechanisms: firstly, by the inhibition of Akt kinase activation, and secondly, by binding to and promoting the degradation of C/EBPβ, the transcription factor required for an early stage of adipogenesis (Naiki *et al.*, 2007).

2.4. The functional roles of TRB3

The human ***Tribbles homolog 3*** (*TRB3*, *Trib3, NIPK, SKIP3, SINK*) gene is located on chromosome 20 at 13-p12.2. *TRB3* was first described as a gene activated during the neuronal cell death induced by the nerve growth factor deprivation and the expose to calcium ionophore A23187 (Mayumi-Matsuda *et al.*, 1999). Further studies have revealed that TRB3 expression is upregulated in many cell types in response to various stresses, including nutrient deficiency, endoplasmic reticulum (ER) stress, hypoxia and oxidative stress (Bowers *et al.*, 2003; Ord and Ord, 2003; Corcoran *et al.*, 2005; Ohoka *et al.*, 2005; Ord and Ord, 2005; Schwarzer *et al.*, 2006; Yacoub Wasef *et al.*, 2006). The overexpression of *TRB3* mRNA has been demonstrated in multiple types of human carcinomas, including breast, lung, colon, esophageal, and ovarian tumors (Bowers *et al.*, 2003; Xu *et al.*, 2007). It is not yet known whether TRB3 has a functional role in tumorigenesis.

TRB3 has been implicated in a wide range of physiological processes, including glucose and lipid metabolism, the preadipocytes differentiation and stress response. One of the main lines of investigations on TRB3 cellular functions deals with the role of TRB3 in insulin signaling and glucose homeostasis. As it is known, insulin is secreted by pancreatic β cells in response to the elevation in the serum glucose level that occurs after eating. Insulin inhibits glucose production in the liver and promotes glucose disposal in the muscle and adipose tissue. Du *et al*. (2003) demonstrated that TRB3 binds to serine/threonine kinase Akt/PKB, the principal target of insulin signaling, preventing its phosphorylation and activation. Furthermore, the enforced expression of TRB3 in cultured cells decreases the activation of Akt by insulin, and in mice, it increases the glucose output from the liver and suppresses the glucose uptake into the muscle, rising the blood glucose level, while the knockdown of TRB3 expression has opposite effects (Du *et al.*, 2003; Koo *et al.*, 2004; He *et al.*, 2006; Koh *et al.*, 2006; Matsushima *et al.*, 2006). Based on these results, it was suggested that TRB3, the expression of which is induced in the mouse liver by fasting, elevates the hepatic glucose output in the conditions of starvation. Importantly, Du *et al*. (2003) proposed that the pathological expression of TRB3 in a fed state may promote hyperglycemia and insulin resistance, and in this way, contribute to the development of type II diabetes. Indeed, TRB3 is upregulated in the liver of a diabetic mouse, and the knockdown of TRB3 expression in a diabetic liver reportedly improves insulin signaling and lowers the glucose level in blood (Du *et al.*, 2003; He *et al.*, 2006; Matsushima *et al.*, 2006). Also, it is relevant to mention that TRB3 mRNA and protein levels are increased in hepatocytes in response to the triglyceride accumulation and the ethanol intoxication, the pathological conditions that support the development of type II diabetes (Klingenspor *et al.*, 1999; Rutter, 2000; Du *et al.*, 2003; He *et al.*, 2006). In addition, the polymorphism of human TRB3 (Q84R) has been found to be associated with insulin resistance (Prudente *et al.*, 2005). However, the pivotal role of TRB3 in the regulation of glucose metabolism has been recently questioned, since TRB3 knockout mice display normal hepatic insulin signaling and glucose homeostasis (Okamoto *et al.*, 2007). Also, when TRB3 is overexpressed in the rat primary hepatocytes, it fails to inhibit the insulin-stimulated activation of Akt (Iynedjian, 2005).

The involvement of TRB3 in lipid metabolism was demonstrated in transgenic mice expressing TRB3 in adipose tissue (Qi *et al.*, 2006). These mice are lean and protected from diet-induced obesity due to increased fatty acid oxidation in adipocytes. In these adipocytes, TRB3 acts as an adaptor protein between acetyl-coenzyme A carboxylase (ACC) and E3 ubiquitin ligase COP1, leading to the enhanced ACC ubiquitination and degradation, and shifting, therefore, the balance of fatty acid metabolism away from lipogenesis to oxidation. Qi *et al*. (2006) suggested that TRB3, the abundance of which is increased during fasting in adipocytes, promotes the lipolysis and fat utilization

during fasting. However, the characterization of the TRB3-deficient mouse revealed no alteration in the ACC level in all tissues analyzed (white and brown adipose tissue, liver, sceletal muscle) and no change in the body fat mass and the serum lipid level (Okamoto *et al.*, 2007). The reason for this discrepancy is currently not known. One possibility is that other genes compensate the lack of TRB3 in the maintenance of lipid homeostasis. Notably, similarly to TRB3, TRB1 is also able to associate with COP1 (Qi *et al.*, 2006).

Several reports have identified TRB3 as a novel anti-adipogenic factor, the expression of which is immediately downregulated when preadipocytes are stimulated to differentiate (Bezy *et al.*, 2007; Naiki *et al.*, 2007; Takahashi *et al.*, 2008). This decline in TRB3 allows the activation of Akt kinase, required for the induction of adipocyte differentiation (Naiki *et al.*, 2007). Also, the downregulation of TRB3 enables C/EBPβ to induce the transcription of proadipogenic genes, such as the peroxisome proliferator-activated receptor γ (PPARγ), the key regulator of adipose tissue lipid metabolism and endocrine function. If overexpressed, TRB3 prevents the phosphorylation of regulatory sites on C/EBPβ and inhibits its DNA-binding ability, and, as a result, suppresses the transcriptional activity of C/EBPβ (Bezy *et al.*, 2007). As described in Chapter 2.3, TRB2 possesses similar anti-adipogenic properties. Distinctly from TRB2, the expression of TRB3 is restored in mature adipocytes. TRB3 binds to PPARγ and inhibits the expression of PPARγ target genes, such as adiponectin, the hormone with anti-hyperglycaemic and anti-inflammatory properties, and perilipin, the critical factor for fat storage and breakdown (Takahashi *et al.*, 2008). Because the forced TRB3 expression decreases and the knockdown of TRB3 expression by RNA interference (RNAi) elevates intracellular triglyceride levels, the authors suggest that TRB3 prevents the abnormal accumulation of fat in adipocytes that is the case in obesity and in other metabolic disorders. One the other hand, the suppression of adiponectin expression by TRB3 may facilitate the development of hyperglycaemia and insulin resistance, as thiazolidinediones, the high-affinity agonist ligands for PPARγ, are clinically effective antidiabetic drugs that improve insulin sensitivity supposedly through upregulating the expression of adiponectin (Lehrke and Lazar, 2005).

Recently, a couple of novel roles of TRB3 have been described. First, TRB3, as the inhibitor of Akt kinase, blocks the differentiation of C2C12 myoblasts into myotubes, and is suggested to regulate the development of muscle cells (Kato and Du, 2007). Second, TRB3 has been identified by Chan *et al*. (2007) as a positive regulator of the bone morphogenetic protein (BMP) signaling pathway. After the ligand-stimulated activation of the BMP pathway, TRB3 dissociates from the BMP type II receptor, and binds to Smad ubiquitin regulatory factor 1 (Smurf1), an inhibitor of the BMP pathway, promoting the degradation of Smurf1. The downregulation of TRB3 inhibits BMP-mediated

cellular responses, including the differentiation of pulmonary artery smooth muscle cells (Chan *et al.*, 2007).

An important investigation area of TRB3 functioning is the characterization of its role in stressed cells. TRB3 has been implicated in the regulation of MAPK, nuclear factor-kappa B (NFκB) and ATF4-CHOP signaling routes, all of which are crucial for the cellular survival in various conditions of stress. It has been reported that TRB3 controls MAPK signaling by binding to MAPK kinases MEK1 and MKK7 (Kiss-Toth *et al.*, 2004), and NFκB signaling by binding to NFκB transactivator p65 (Wu *et al.*, 2003). The interaction of TRB3 with p65 results in the inhibition of p65 phosphorylation, and in the suppression of NFκB-dependent transcription, leading consequently to the reduced NFκB-dependent cell survival. The role of TRB3 as a regulator of the ATF4-CHOP signaling pathway will be described below in Chapter 2.6.

2.5. The structural properties and developmental functions of ATF4

ATF4, also called cyclic AMP response element-binding protein 2 (CREB2), is a member of the ATF/CREB subfamily of basic region-leucine zipper (bZip) transcription factors, which have the consensus binding site cAMP responsive element (CRE). The other members of the ATF/CREB subfamily include CREB, CREM, ATF1, ATF2, ATF3, ATF5, and ATF6. The human ATF4 protein consists of 351 amino acids, and is structured into N-terminally located transcription activation domain (Schoch *et al.*, 2001) and C-terminally located bZip domain, wherein the basic amino acid-rich DNA-binding region is followed by the leucine zipper dimerization motif (Fig. 2).

Binding of ATF4 to specific DNA sequences requires homo- or heterodimerization. ATF4 has been demonstrated to heterodimerize with a large set of bZip transcription factors, including Jun, Fos, Fra-1 (Hai and Curran, 1991) and JunD (Shimizu *et al.*, 1998) from the AP-1 subfamily; C/EBPβ (Vallejo *et al.*, 1993; Podust *et al.*, 2001) and C/EBP homologous protein (CHOP) (Gachon *et al.*, 2001) from the C/EBP subfamily; and NF-E2 related factor 2 (Nrf2) from the Cap'n'Collar-bZip subfamily (He *et al.*, 2001). The heterodimerization of ATF4 modulates the DNA binding specificity and affects the output of target genes. For instance, ATF4 in complex with its own subfamily members activates *via* the consensus CRE sequence (5'-TGACGTCA-3') the transcription of *growth arrest-and DNA damage-inducible gene 34* (*GADD34*) (Ma and Hendershot, 2003) and *via* the CRE-like sequence (5'-TGACGTGA-3') the transcription of *glucose-regulated protein 78* (*grp78*) gene (Luo *et al.*, 2003). ATF4 dimerized with Nrf2 binds to the stress-response element (5'-T/CGCTGAGTCA-3') in *heme oxygenase-1* promoter (He *et al.*, 2001). ATF4 in complex with cJun is able to bind to the ATF/AP-1 sequence (5'-TGACGTAA-3') and induce the DNA repair

enzyme *apurinic endonuclease 1* (*Ape1*) transcription (Fung *et al.*, 2007). The majority of currently characterized target genes of ATF4 are activated by the dimers of ATF4 and C/EBP subfamily members *via* the C/EBP-ATF composite site (5'-R/TTTG/TCATCA-3', R = G or A) (Fawcett *et al.*, 1999), also known as the amino acid response element (AARE) in the *CHOP* promoter (Averous *et al.*, 2004) and the nutrient stress response element-1 (NSRE-1) in the *asparagine synthetase* (*Asns*) promoter (Siu *et al.*, 2002). The other genes induced through the C/EBP-ATF site by ATF4 include *TRB3* (Ohoka *et al.*, 2005; Ord and Ord, 2005), the *vascular endothelial growth factor* (*VEGF*) (Roybal *et al.*, 2005), the *insulin-like growth factor binding protein-1* (*IGFBP-1*) (Marchand *et al.*, 2006), *osteocalcin* (Yang *et al.*, 2004), and *presenilin-1* (Mitsuda *et al.*, 2007). Further, the identification of ATF4 target genes using the microarray analysis established that ATF4 is a master regulator of the cellular amino acid metabolism by activating the bulk of genes encoding enzymes involved in amino acid biosynthesis, amino acid transporters, and aminoacyl-tRNA synthetases (Harding *et al.*, 2003; Adams, 2007). Most likely, the ATF4-dependent regulation of this group of genes is also mediated through the C/EBP-ATF site, because the site has been found upstream of the transcription start site of these genes by computer search (Adams, 2007). Experimental studies have already verified this assumption in the case of *cystine/glutamate transporter* (Sato *et al.*, 2004), *arginine/lysine transporter* (Fernandez *et al.*, 2003) and *Asns* mentioned above.

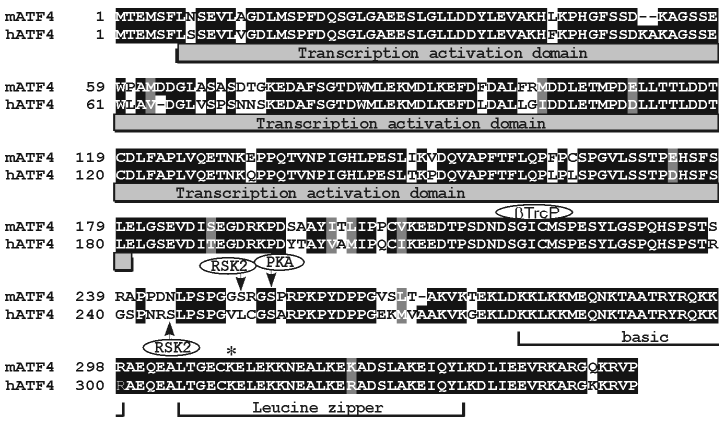

Figure 2. Alignment of the amino acid sequences of mouse ATF4 (mATF4; the Swiss-Prot/TrEMBL database accession number Q06507) and human ATF4 (hATF4; P18848). The transcriptional activation domain (Schoch *et al.*, 2001) and the bZip domain with the basic and leucine zipper region are indicated. The binding site of βTrCP, the component of SCF E3 ubiquitin ligase, resides at positions 218 to 224 of hATF4. Arrowheads point to the serines phosphorylated by ribosomal protein S6 kinase 2 (RSK2) and protein kinase A (PKA). The acetylation site for the histone acetyltransferase p300 is marked by an asterisk.

The transcriptional activating activity of ATF4 is modulated by direct protein-protein interactions and by posttranslational modifications. ATF4 has been demonstrated to interact with general transcription factors such as TATA-binding protein, TFIIB, the RAP30 subunit of TFIIF (Liang and Hai, 1997) and with RNA polymerase II subunit 3 (De Angelis *et al.*, 2003). ATF4 also interacts with several transcriptional coactivators, such as the CREB-binding protein (CBP), its homolog p300 and the p300/CBP-associated factor (PCAF), which stimulate the ATF4-driven transcription (Liang and Hai, 1997; Yukawa *et al.*, 1999; Lassot *et al.*, 2005; Cherasse *et al.*, 2007). The inhibitors of ATF4 that bind to its transactivation domain, like pseudokinase TRB3, may impede the association of transriptional coactivators to the same domain, and therefore suppress the transcriptional activation activity of ATF4 (Ord and Ord, 2003; Ohoka *et al.*, 2007). Other known repressors of ATF4 transcriptional activity, such as the nuclear matrix protein mitosin/CENP-F, factor inhibiting ATF4 (FIAT) and latency-associated nuclear antigen (LANA) contain a leucine zipper motif by which they heterodimerize with the bZip domain of ATF4 and thereby prohibit the binding of ATF4 to its cognate DNA sequence (Lim *et al.*, 2000; Zhou *et al.*, 2005; Yu *et al.*, 2005).

The modulation of ATF4 protein stability constitutes an important mechanism to control its protein level and activity. ATF4 is an unstable protein (Vallejo *et al.*, 1993) that is rapidly degraded *via* a ubiquitin-proteasomal pathway. ATF4 contains a recognation motif DSGXXXS for the E3 ubiquitin ligase $SCF^{\beta TrCP}$ (Fig. 2). The phosphorylation of the first serine of this motif leads to the binding of ATF4 to $SCF^{\beta TrCP}$, followed by ATF4 ubiquitination and consequent degradation by the proteasome (Lassot *et al.*, 2001). Differently from non-osteoblastic cells, ATF4 is protected from degradation in osteoblasts, resulting in ATF4 accumulation and increased expression of ATF4 target genes (Yang and Karsenty, 2004). The recent studies (Lassot *et al.*, 2005; Yu *et al.*, 2008) have revealed that ATF4 can be stabilized through direct interactions with coactivator p300 and the general transcription factor TFIIAγ that both markedly increase the level of ATF4 protein and the ATF4-dependent transcriptional activity by inhibiting its ubiquitination and proteasomal degradation. Although p300 can also acetylate ATF4 within bZip region (Gachon *et al.*, 2002), p300-mediated ATF4 stabilization is independent of its acetyltransferase activity (Lassot *et al.*, 2005).

ATF4 contains a strong transcription activation domain that is suggested to be constitutively active (Schoch *et al.*, 2001; Thiel *et al.*, 2005). However, phosphorylation seems to be critical for ATF4 to elicit its function in osteoblasts and bone. Yang *et al*. (2004) showed that the phosphorylation of ATF4 by ribosomal protein S6 kinase 2 (RSK2) is required for the expression of *osteocalcin* gene and the onset of osteoblast differentiation. Furthermore, the phosphorylation of ATF4 by RSK2 enhances its activity and subsequent amino acid import into osteoblasts, thereby facilitating type I collagen synthesis and

bone formation/mineralization. The lack of ATF4 phosphorylation by RSK2 is thought to contribute to the skeletal phenotype of the Coffin-Lowry syndrome, a genetic disorder caused by RSK2 deficiency and characterized by a block in the differentiation of osteoblasts and diminished bone mineralization, which are the defects observed also in the RSK2 knockout mouse as well as in the ATF4 knockout mouse (Yang *et al.*, 2004). Protein kinase A (PKA)-dependent phosphorylation of ATF4 is similarly stimulative to its activity in osteoblasts and leads to the increased amino acid import and enhanced bone formation (Elefteriou *et al.*, 2006). But, additionally, PKA-phosphorylated ATF4 activates the expression of the *receptor activator of NF-κB ligand* (*RANKL*) that induce the differentiation of osteoclast involved in bone destruction. Because the increased bone formation with simultaneously elevated bone destruction (resulting in the bowed bones) is the characteristic feature of the genetic disorder neurofibromatosis type I (NF1), and the mice overexpressing ATF4 in their osteoblasts display the skeletal phenotype similar to NF1, it was suggested that an excessive phosphorylation of ATF4 by PKA may be causative to the pathogenesis of NF1 (Elefteriou *et al.*, 2006). Strikingly, both ATF4-phosphorylation-dependent skeletal defects described are treatable by dietary manipulations: a low-protein diet normalizes bone formation in the NF1 mouse model, and a high-protein diet overcomes developmental defects and low bone mass in the ATF4-deficient mouse and in the RSK2-deficient mouse (Elefteriou *et al.*, 2006). These results indicate that one of the primary functions of ATF4 is to control an amino acid metabolism that, in turn, affects cell growth, differentiation and tissue development.

Several studies (Tanaka *et al.*, 1998; Hettmann *et al.*, 2000; Masuoka and Townes, 2002) report that ATF4 knockout mice are blind, anemic, and growth retarded due to the defective lens development, the reduced proliferation of hematopoietic progenitors, and the skeletal defects mentioned above, respectively. The overexpression of ATF4 in the mammary gland of the transgenic mouse leads to the decreased proliferation, impaired differentiation and the increased apoptosis of mammary epithelium (Bagheri-Yarmand *et al.*, 2003). Thus, ATF4-dependent gene regulation is essential for the normal proliferation and differentiation processeses in mammalian development, whereas the activity of ATF4 must be under tight cellular control, because either too much or too little ATF4 is detrimental to cellular functions. Another major function carried out by ATF4 is the orchestration of gene expression in response to a variety of unfavorable conditions, as will be described next.

2.6. The role of ATF4 in cellular stress response

An important physiological role of ATF4 is to regulate cell response to various stress signals, such as the accumulation of incorrectly folded proteins in endoplasmic reticulum, oxygen and nutrient deprivation, changes in redox homeostasis, heme deficiency, and viral infection. Under these adverse conditions, the translation of ATF4 mRNA is selectively increased, although the general translation is attenuated due to the phosphorylation of alpha subunit of eukaryotic translation initiation factor 2 (eIF2α), leading to the inhibition of eIF2 recycling to its active GTP-bound form (Wek *et al.*, 2006). The phosphorylation of eIF2α is carried out by one of four mammalian eIF2α kinases, PERK, PKR, GCN2, and HRI, each of which can be activated by a specific set of stress conditions: the ER transmembrane kinase PERK is activated in response to the malfunction of ER, the cytoplasmic kinase PKR is activated by double-stranded RNA and helps protect the cell from viral infection, the GCN2 functions as a cytoplasmic sensor of amino-acid levels and the HRI is a heme-regulated inhibitor that is predominantly expressed in erythroid cells (Fig. 3) (Proud, 2005). The 5' untranslated region (5' UTR) of ATF4 mRNA contains 2 upstream open reading frames (uORFs) that regulate translation (Lu *et al.*, 2004; Vattem and Wek, 2004). Under non-stressed conditions, the reinitiation of translation at uAUG2 by scanning ribosomes precludes the translation of ATF4 ORF. The phosphorylation of eIF2α during stress delays translation reinitiation, allowing scanning ribosomes to bypass the inhibitory uORF2, and instead translate ATF4 coding region, resulting in the rapid elevation of ATF4 protein production. ATF4 plays a major role in the control of gene expression during the integrated stress response (ISR), the adaptive response to divergent cellular stresses mediated by signaling pathways that converge on a single event – the phosphorylation of eIF2α (Fig. 3) (Harding *et al.*, 2003).

Among ISR branches, the pathway conducted by PERK is one of the best-studied. As mentioned above, PERK is activated in the conditions of ER stress caused by the accumulation of misfolded proteins in the ER that can happen with mutant proteins in pathophysiological conditions. But this can also occur under normal physiological conditions, especially in the cells with highly active synthesis of secretory proteins, like the insulin-secreting pancreatic β cells, the immunoglobulin-secreting plasma cells and collagen-producing osteoblasts. In experimental models, the ER stress is evoked by pharmacological inducers such as tunicamycin and thapsigargin, which cause the inhibition of N-linked glycosylation and the disruption of calcium homeostasis, respectively. To alleviate the ER stress, two other ER-resident stress sensors, the transcription factor ATF6 and the transmembrane kinase/endoribonuclease IRE1 (inositol-requiring enzyme 1), are activated to cooperate with PERK for the initiation of coordinated stress response called the unfolded protein response (UPR) (Rutkowski and Kaufman, 2004; Schroder and Kaufman, 2006) (Fig. 3). One of

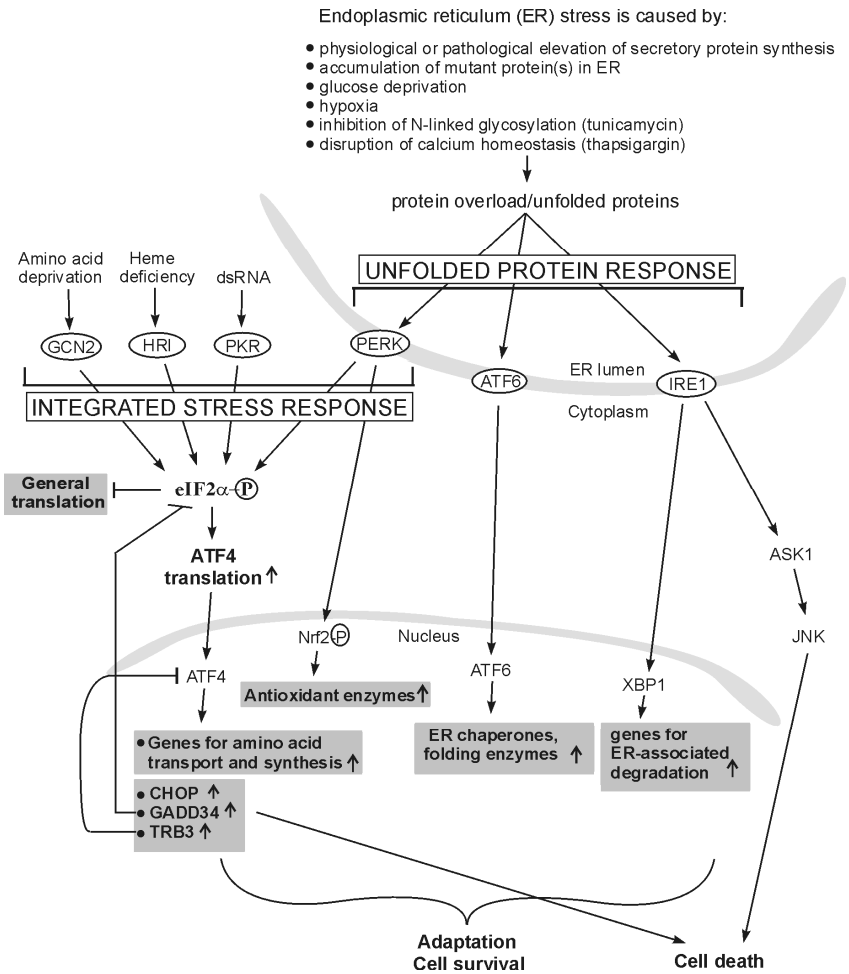

Figure 3. Transcription factor ATF4 is a crucial component of integrated stress response (ISR) and endoplasmic reticulum (ER) stress response/unfolded protein response (UPR). The perturbation of ER homeostasis causes the ER stress due to the accumulation of unfolded proteins in the lumen of ER. The signaling of UPR is simultaneously initiated by three ER-transmembrane proteins: PKR-like ER kinase (PERK), transcription factor ATF6 and inositol-requiring enzyme 1 (IRE1). The phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) by PERK suppresses the general protein synthesis, but increases the translation of ATF4. Because three other eIF2α kinases (GCN2, HRI and PKR) lead to the same translational consequences in cells suffering from different stresses (amino acid insufficiency, heme deficiency and the presence of double-stranded (ds)RNA, respectively), the eIF2α-ATF4 pathway functions more broadly than UPR to direct ISR. ATF4 upregulates the transcription of genes involved in amino acid transport and synthesis. ATF4 also upregulates the expression of genes involved in stress response signaling such as CHOP, a proapoptotic transcription factor, GADD34, a regulatory subunit of

phosphatase PP1 that dephosphorylates eIF2α and terminates signaling in the ISR, and TRB3, a pseudokinase that inhibits ATF4 transcriptional activity. The phosphorylation of transcription factor Nrf2 by PERK promotes Nrf2 nuclear entry followed by the transcription of genes encoding the antioxidant enzymes that support redox homeostasis. In UPR, ATF6 activates the transcription of genes encoding ER chaperones and folding enzymes that augment the folding capacity of ER. IRE1 increases the expression of transcription factor XBP1 that in turn induces the transcription of genes involved in protein degradation to diminish the amount of unfolded proteins in ER. In most circumstances, UPR (and ISR) signaling promotes adaptation and cell survival. However, the ER stress exceeding a certain threshold triggers the cell death program by strongly upregulated ATF4-CHOP-GADD34 pathway and/or by IRE1-mediated activation of apoptosis signaling kinase 1 (ASK1) and cJun N-terminal kinase (JNK).

the earliest events in the UPR is the PERK-mediated phosphorylation of eIF2α that causes immediate, yet transient, shutdown of general translation and reduces in this way the protein influx to ER. The next step of UPR is conducted by the activation of transcription factors, such as ATF4, Nrf2, ATF6, and XBP1, which in turn upregulate various stress responsive genes needed for resolving the stress situation. The activation of ATF6- and IRE1/XBP1-dependent branches of UPR induces the transcription of genes encoding ER chaperones and folding enzymes, and the genes involved in protein degradation, that will collectively reduce the unfolded protein load in ER (Schroder and Kaufman, 2006; Yamamoto *et al.*, 2007a). Thus, these two pathways of UPR deal with resolving specifically the ER stress, while the PERK-dependent activation of ATF4 and Nrf2 results in the upregulation of genes that regulate more general cellular processeses, such as amino acid metabolism and redox homeostasis, respectively (Harding *et al.*, 2003; Cullinan and Diehl, 2004).

UPR, as a protective response, results in the adaptation and restoration of cellular homeostasis. The importance of functional eIF2α-ATF4 pathway for cell survival has been demonstrated by the increased sensitivity of PERK-deficient cells to glucose starvation (Scheuner *et al.*, 2001) and ATF4-deficient cells to amino acid depletion and oxidative stress (Harding *et al.*, 2003). However, a sufficiently severe and prolonged ER stress can trigger the ER-mediated proapoptotic signaling, involving IRE1 and/or ATF4 pathways (Fig. 3). For instance, the activated IRE1 can contribute to the induction of apoptosis either by the activation of apoptosis signal-regulating kinases ASK1 and JNK (Urano *et al.*, 2000) or by direct interaction with the Bcl-2 family proapoptotic members Bax and Bak (Hetz *et al.*, 2006). In ATF4-dependent signaling, the crucial proteins involved in the switching of UPR from adaptation to suicide are CHOP, GADD34 and TRB3. These proteins, as will be discussed below, may affect cellular sensitivity to the ER stress in opposing ways. The general consideration is that the enhanced level of CHOP in UPR promotes cell death, since CHOP deficiency protects mouse embryonic fibroblasts (MEF) from the

ER stress-induced apoptosis (Zinszner *et al.*, 1998). At the same time, the protective role of CHOP in oligodendrocytes suffering from the ER stress has also been described (Southwood *et al.*, 2002). CHOP is suggested to evoke apoptosis by several mechanisms, including the downregulation of anti-apoptotic gene *Bcl-2* expression (McCullough *et al.*, 2001), the upregulation of the death receptor family member DR5 (Yamaguchi and Wang, 2004), and the ability to change ER redox conditions by inducing the transcription of ER oxidase *Ero1* (Marciniak *et al.*, 2004). Furthermore, according to a recent report, CHOP activates the expression of *GADD34* (Marciniak *et al.*, 2004). In addition to CHOP, the expression of *GADD34* is induced by ATF4 (Ma and Hendershot, 2003). GADD34 binds to the catalytic subunit of protein phosphatase 1 (PP1) and promotes the dephosphorylation of eIF2α and thereby the release of the global translational block (Novoa *et al.*, 2001; Marciniak *et al.*, 2004). The perfect timing of the GADD34 expression during UPR (and ISR) is crucial in the determination of the cell fate. While it is clear that the GADD34-mediated resumption of protein synthesis is essential for the expression of UPR target genes that support the recovery from the ER stress (Ma and Hendershot, 2003), the enhancement of protein synthesis during a severe ER stress augments further the ER overload and can ultimately lead to cell death. The pro-apoptotic role of GADD34 is demonstrated in the mice lacking GADD34-directed eIF2α dephosphorylation: the loss of GADD34 phosphatase activity protects the renal epithelium from tunicamycin-induced apoptosis (Marciniak *et al.*, 2004). Also, ectopically expressed GADD34 is growth-inhibitory and proapoptotic (Hollander *et al.*, 2001). Importantly, one of the consequences of GADD34 expression is the downregulation of ATF4 translation and, therefore, GADD34 functions as a negative feedback regulator of the ATF4 pathway and represses the expression of ATF4 target genes, including proapoptotic CHOP (Fig. 3) (Novoa *et al.*, 2001). Another negative feedback regulator of the ATF4 pathway is TRB3 that interacts with and inhibits the transcriptional activity of ATF4 (Bowers *et al.*, 2003; Ord and Ord, 2003; Ord and Ord, 2005; Jousse *et al.*, 2007). Ohoka *et al.* (2005) have reported that *TRB3* is also the target gene for CHOP, and moreover, TRB3 is able to reduce the transactivation activity of CHOP by binding directly to CHOP. The enforced TRB3 expression does not affect the growth and viability of cells in normal conditions (Ord *et al.*, 2007). The currently published data suggest that TRB3 may have different effects on the survival of stressed cells, depending on the inducer of stress and/or cellular context. The knockdown of TRB3 through RNA interference confers resistance against tunicamycine in HEK293T and HeLa cells (Ohoka *et al.*, 2005), but accelerates death in glucose-deprived SaOS2 cells (Ord *et al.*, 2007). Consistent with the latter finding, the overexpression of TRB3 protects PC-3 prostate carcinoma cells against glucose deprivation-induced apoptosis (Schwarzer *et al.*, 2006).

Thus, CHOP, GADD34 and TRB3 are crucial components of ATF4 pathway controlling the transition of ATF4-mediated prosurvival response to apoptosis. At present it is not entirely clear when and why the protective ATF4 pathway in UPR develops to the death-inducing pathway. There is no doubt that the certain threshold level of stress is important for the transition of the prosurvival responses to apoptosis. For example, in osteoblasts, the mild ER stress evoked by a short term (1 h) thapsigargin or tunicamycine treatment favors the transcriptional activation of the ATF4-dependent genes participating in bone remodeling, while the prolonged treatment (for 24 h) induces apoptosis (Hamamura and Yokota, 2007). It has also been suggested that the cell survival under a mild ER stress is facilitated by the higher intrinsic instability of cell-death mediators (such as CHOP and GADD34) compared to that of ER chaperons promoting adaptation (like grp78) (Rutkowski *et al.*, 2006). According to this model, the level of apoptotic proteins exceeds the death threshold only after a prolonged and severe ER stress. Finally, it is possible that the ATF4-dependent stress response may be greatly affected by the eIF2α kinase activated in the cell. As recently demonstrated, not only PERK, but also PKR is activated in response to the ER stress, whereas the PKR knockdown, but not the PERK knockdown, reduces markedly thapsigargin-induced eIF2α-ATF4-CHOP signaling and the cell death associated to this (Lee *et al.*, 2007). Similarly, the inhibition of PKR activation protects retinal neurons against the ER-stress induced damage (Shimazawa *et al.*, 2007). The difference between PERK- and PKR-mediated cellular responses might be attributable to the different substrates of these kinases, besides a common substrate eIF2α. In this context, PERK supposedly promotes cell survival rather than cell death as the result of the induction of survival factors (like Nrf2, which increases the level of antioxidants during stress) (Cullinan and Diehl, 2004). On the contrary, PKR is more probably involved in cell death *via* the activation of eIF2α-ATF4 pathway simultaneously with proapoptotic factors, like the p38 mitogen-activated protein kinase, which has been shown to enhance also the proapoptotic properties of CHOP (Wang and Ron, 1996; Maytin *et al.*, 2001; Scheuner *et al.*, 2006).

In many instances, the perturbation of ER homeostasis has been correlated with pathologic states, particularly with diabetes, cancer, ischemia and neuro-degenerative diseases. The functional eIF2α-ATF4 pathway has been implicated in the pathogenesis as well as in the curing of these illnesses depending on the situation. For example, exendin-4, the drug administrated against the progressive loss of insulin-secreting β cells during the development of type II diabetes, is the incretine-related peptide hormone, which, by upregulating specifically the translation of ATF4, induces GADD34 and releases the block of protein synthesis and thereby increases the production of insulin and β-cell survival (Yusta *et al.*, 2006). Salubrinal, a small-molecule inhibitor of eIF2α dephosphorylation that specifically inhibits GADD34-dependent phosphatase activity and leads to the sustained eIF2α phosphorylation in conditions of stress,

has been demonstrated to be useful against viral infection (Boyce *et al.*, 2005). At the same time, the salubrinal-induced expression of ATF4, and concomitantly CHOP, increases the fatty acid-induced ER stress and apoptosis in pancreatic β cells (Cnop *et al.*, 2007). The involvement of CHOP in the pathogenesis of the ER stress-related diseases has been confirmed by the observation that the deletion of CHOP protects β cells from malfolded insulin in an Akita mouse model of diabetes mellitus (Oyadomari *et al.*, 2002) and dopaminergic neurons in an in vivo neurotoxin model of the Parkinson's disease (Silva *et al.*, 2005). Further, the data from the mouse models of brain ischemia have revealed the connection between the elevated level of ATF4 and CHOP proteins and the apoptosis in ischemic hippocampal neurons (Tajiri *et al.*, 2004; Hayashi *et al.*, 2005). The increased ATF4 expression has been implicated as a potential pathogenic factor in the neurological disorder called vanishing white matter disease (Kantor *et al.*, 2005; van der Voorn *et al.*, 2005). In the cancer-related pathology, the sustained activity of the ATF4 pathway confers a survival advantage for tumor cells under hypoxia (Bi *et al.*, 2005). Potential anti-tumoral therapies in that case involve the magnification of the ER stress in cancer cells to the level that activates the apoptotic signaling of UPR. For instance, the proteasome inhibitor PS-341 (Bortezomib) that has been approved for treating human malignancies induces apoptosis ATF4-CHOP pathway-dependently (Jiang and Wek, 2005; Fribley *et al.*, 2006; Obeng *et al.*, 2006). Also, the activation of the ATF4 pathway in pancreatic tumor cells by cannabinoid treatment promotes apoptosis, and the downregulation of either ATF4 or TRB3 mRNA levels reduces cancer cell death (Carracedo *et al.*, 2006a; Carracedo *et al.*, 2006b). Thus, the stress response pathway of ATF4 plays a significant role in the determination of the cell fate and is often a crucial factor in pathophysiological conditions. Therefore, understanding the mechanisms directing the ATF4 pathway may help to find a way to treat or halt the progress of such conditions.

3. RESULTS AND DISCUSSION

3.1. Objectives of the present study

The starting point of this project was when our group identified by differential cloning a gene, currently known as *TRB3*, that is strongly activated in neuronal cells suffering from disturbed calcium metabolism to the extent that eventually leads to cell death. At that time, no information concerning TRB3 had been published, and, therefore, the characterization of the gene was initiated. The objectives of the present study are:

1. To characterize the mechanism of the stress-dependent transcriptional activation of *TRB3* gene;
2. To identify proteins interacting with TRB3;
3. To study the functional role of TRB3 in the cellular stress response.

3.2. The gene encoding pseudokinase TRB3 (NIPK) is upregulated in response to various cellular stresses (I, II)

The perturbation of Ca^{2+} homeostasis has been associated with the death of neurons in several neuropathological states such as ischemia and neurodegenerative disordes (Mattson *et al.*, 2000). To study the changes in the gene expression pattern caused by the disruption of Ca^{2+} homeostasis, we exposed the mouse hypothalamic neuronal GT1-7 cells to thapsigargin, an inhibitor of endoplasmic reticular Ca^{2+}-ATPase, which, as previously reported (Wei *et al.*, 1998), induces the apoptotic death of GT1-7 cells. We carried out the PCR-coupled subtractive cDNA cloning procedure RDA (Hubank and Schatz, 1994), and identified a gene (*RDA-54*) strongly activated in thapsigargin-treated GT1-7 cells (I, Fig. 1A). A 1280 bp cDNA (the GenBank database accession number AJ514260), covering the full coding region of *RDA-54* (354 amino acids), was isolated from the mouse Marathon-Ready cDNA (Clontech), and the nucleotide sequence analysis revealed that *RDA-54* represents the mouse homolog of the rat gene *NIPK* (*neuronal cell death-inducible putative kinase*) (Mayumi-Matsuda *et al.*, 1999). Therefore, RDA-54 was designated by us initially as the mouse NIPK, and currently the name NIPK has been replaced by TRB3 (Tribbles homolog 3). The mouse TRB3 (mTRB3) contains a region similar to the protein kinase domain and has 92%, 48% and 37% sequence identity to the rat TRB3 (NIPK), the canine TRB2 (c5fw) (Wilkin *et al.*, 1997) and *Drosophila* Tribbles (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000), respectively (I, Fig. 2). As described in the review of literature (Chapter 2.1), it is likely that TRB3 and other Tribbles family members have no kinase

catalytic activity due to the substitution of several crucial amino acid residues, and, therefore, they are called pseudokinases or kinase-like proteins.

To determine the tissue distribution of *TRB3*, we used a commercial Mouse Multiple Tissue Northern Blot (Clontech), and found that ~ 2 kb *TRB3* mRNA is abundantly present in the adult mouse liver, but scarce or undetectable in heart, brain, spleen, lung, skeletal muscle, kidney, and testis (I, Fig. 1B). Consistently, Bowers *et al*. (2003) reported that the normal tissue expression of human *TRB3* (*hTRB3*) mRNA is similarly confined to liver with a very low expression in other tissues. The timecourse analysis of the stress-induced expression of *TRB3* by the Northern blot demonstrated that in the GT1-7 cells exposed to thapsigargin, the upregulation of *TRB3* mRNA starts after 2 h treatment and increases more than 20-fold by 20 h (I, Fig. 5A upper panel). Other groups have detected a similar lag of about 2-6 h prior to a significant increase in the *TRB3* mRNA content in the cells treated by tunicamycin or deprived by nutrients (Corcoran *et al.*, 2005; Ohoka *et al.*, 2005; Schwarzer *et al.*, 2006). It is important to mention that in the same conditions, the significant accumulation of ATF4 protein (which is a good indicator of the activation of ISR) is observed starting from 1 h timepoint (I, Fig. 5B) (Harding *et al.*, 2000; Ohoka *et al.*, 2005). The inducibility of *TRB3* was also studied in the human hepatoblastoma cell line HepG2. *TRB3* is expressed at the level detectable by the Northern blot in the hepatoblastoma cells cultured under normal conditions, and similarly to our finding in GT1-7 cells, the level of *TRB3* mRNA in HepG2 cells is greatly increased by thapsigargin, the inducer of ER stress (II, Fig. 1) (Wong *et al.*, 1993). In addition, sodium arsenite, the toxicity of which is primarily connected to oxidative stress (Kessel *et al.*, 2002), was also tested and found to upregulate the expression of *TRB3* efficiently (II, Fig. 1). To date, it is confirmed that TRB3 is a stress-inducible gene upregulated in cells, as already mentioned, in response to ER stress, oxidative stress and nutrient deficiency, and, in addition, in response to ethanol intoxication and hypoxia (Bowers *et al.*, 2003; He *et al.*, 2006). Interestingly, multiple primary human lung, colon, and breast tumors express high levels of *TRB3* mRNA (Bowers *et al.*, 2003; Xu *et al.*, 2007). It is possible that the tumor-specific TRB3 upregulation is also related to stress, because malignant growth often occurs in the hypoxic and nutrient-limited microenvironment.

3.3. Human *TRB3* mRNA exists in several isoforms differing in the 5' untranslated region (II)

To study the molecular mechanisms of *TRB3* gene regulation during stress, we started with the mapping of the transcription initiation site(s) of *hTRB3* transcripts in stressed cells. We synthesized a cDNA pool from the RNA of thapsigargin-treated HepG2 cells, using the RNA ligase-mediated oligo-capping

method (Maruyama and Sugano, 1994). The method results in the ligation of an RNA oligonucleotide to the 5' ends of full-length mRNA as a PCR primer docking site, ensuring subsequent amplification of only those cDNA molecules that contain intact 5' ends of transcripts. The direct sequencing of 53 clones revealed the presence of several *hTRB3* mRNA isoforms that encode a common protein, but differ in their 5' untranslated region (5' UTR). The 5' UTRs of *hTRB3* mRNA are the alternative variants of the first exon named 1A and 1B (II, Fig. 2A, B). The analysis revealed that exon 1A has a single splice donor site, but the previously undescribed exon 1B generates four alternative splice donor sites. The heterogenity of *hTRB3* transcripts is further increased by the large number of transcription initiation sites used. In exon 1A we identified 10 transcription start sites distributed over the region of about 300-bp and in exon 1B, 9 transcription start sites distributed over the region of about 50-bp (II, Fig. 2B). The cDNA sequences of the longest *hTRB3* isoform 1A and four distinct 5' UTR variants of isoform 1B have been submitted to the GenBank (accession numbers AJ697936-AJ697940). The screening of the human Expressed Sequence Tag (EST) database reveals that additional variants of *hTRB3* mRNA 5' UTR which are not described in our article (II) exist: the database contains the EST clones sequences extending further upstream from the longest exon 1A transcript described by us and an EST clone sequence with a novel splice donor site of exon 1B.

In order to estimate the relative abundance of *hTRB3* mRNA molecules with different 5' UTR in normal conditions and under the stress, we used the Northern hybridization of HepG2 RNA to the probes specific to various regions of *hTRB3* exon 1A and to exon 1B. The 5' UTRs originating from exon 1A have sizes from about 70-bp to more than 400-bp, and the longest representatives of this group contain two uORFs and repeated sequences (II, Fig. 2B). We found that in normal growth conditions, the long exon 1A isoforms are the predominant mRNA variants in cells, and exon 1B isoforms remain below the level of detection, whereas in cells exposed to thapsigargin or arsenite, the *hTRB3* mRNAs containing 1B or short 1A sequences are strongly induced (II, Fig. 2C). The observation that *hTRB3* mRNA molecules synthesized in the cells exposed to thapsigargin are shorter than those synthesized in normal conditions has also been made by other researchers (Corcoran *et al.*, 2005). Since the structural features of mRNA 5' UTR have a major impact on translation initiation, it seems likely that *hTRB3* mRNA isoforms are translated with different efficiency, and, therefore, the changes in the relative abundance of *hTRB3* isoforms may contribute to the regulation of TRB3 protein expression. This issue needs to be addressed in future studies.

3.4. The stress responsiveness of the *hTRB3* gene is mediated by the promoter region containing C/EBP-ATF composite sites (II)

To determine the stress-responsive promoter region of the human *TRB3* gene, we isolated a human genomic DNA fragment extending 0.9-kb upstream from the transcriptional start site of exon 1A that is used most frequently in the conditions of stress (II, Fig. 2B). We assessed the ability of this region to induce the expression of the luciferase reporter gene in the HepG2 cells that were exposed to thapsigargin or arsenite or left unexposed. Since each tested condition resulted in the elevation of luciferase activity, however, the elevation was higher in the stressed cells compared to the unstressed cells, the experiment showed that the isolated *hTRB3* gene fragment indeed contains the promoter elements for basal, as well as for thapsigargin- and arsenite-induced transcription (II, Fig. 3A). Importantly, this *hTRB3* promoter region includes three identical tandemly arranged 33-bp-long repeats, the middle part of which is similar to a regulatory element in the *Chop* promoter, termed C/EBP-ATF composite site (Fawcett *et al.*, 1999) (named also amino acid response element (Bruhat *et al.*, 2000)), and to a regulatory element in the *Asns* promoter, termed nutrient-sensing response element (NSRE)-1 (Barbosa-Tessmann *et al.*, 2000) (II, Fig. 3B). Both these elements are involved in the activation of genes in response to various stresses, including the ER and arsenite stresses (Fawcett *et al.*, 1999; Barbosa-Tessmann *et al.*, 2000; Bruhat *et al.*, 2002; Ma *et al.*, 2002; Averous *et al.*, 2004). We found by the deletion analysis of the *hTRB3* promoter that the sequence consisting of three 33-bp repeats stimulates the luciferase expression in response to thapsigargin and arsenite, while neither regions upstream nor downstream of the 33-bp repeats demonstrate the stress-dependent inducibility of the reporter gene expression (II, Fig. 3A). Thus, the region consisting of tandemly arranged 33-bp repeats is necessary and sufficient for the thapsigargin and the arsenite responsiveness of *hTRB3* promoter.

To map exactly which regions of the 33-bp repeat are involved in the stress-induced *hTRB3* promoter activation, we performed luciferase assays in HepG2 cells, using the reporter constructs driven by the single 33-bp repeat or its mutants. As expected, both thapsigargin and arsenite induce the luciferase expression in the case of wild-type 33-bp repeat, and responsiveness to these agents is lost by the mutation of the ATF part as well as the C/EBP part of the C/EBP-ATF composite site (II, Fig. 3C). In addition to that, mutations in the regions preceding and following the C/EBP-ATF site also affect luciferase activity, inhibiting or stimulating it, respectively (II, Fig. 3C). The mechanisms by which the mutations in the regions flanking the C/EBP-ATF composite site affect the expression of the reporter gene are currently obscure. It is possible that the stimulating properties of the mutated 3' part of 33-bp repeat are a result of the loss

of a binding site of a transcriptional repressor. Alternatively, the increased responsiveness to stress may be caused by the enhanced binding of the positive regulator(s) of transcription to the mutated 3' flanking region. Our data indicate that the C/EBP-ATF composite site as well as the intact 5' part of 33-bp repeat are both required for the thapsigargin- or arsenite-dependent activation of the *hTRB3* promoter. The results are in good agreement with the observations made by Ohoka *et al*. (2005) that both the C/EBP-ATF site and its preceding sequence are required for the induction of the *hTRB3* promoter by tunicamycin, the compound that evokes the ER stress by inhibiting N-linked glycosylation.

Taking together, the stress-dependent induction of *hTRB3* gene is mediated by the promoter region consisting of 33-bp repeats that contain the C/EBP-ATF composite site. Consistent with this finding, Selim *et al*. (2007) recently reported that the nucleotide sequence similar to the 33-bp repeat of the *hTRB3* promoter is present as a unique, one-copy motif in the 5' flanking region of the mouse *TRB3* gene and participates in the activation of the gene in response to stress.

3.5. ATF4 activates the *TRB3* promoter by binding to the C/EBP-ATF composite site (II, III)

Previous studies have demonstrated that the stress-responsive activation of *Chop* promoter through C/EBP-ATF composite site and *Asns* promoter through NSRE-1 is mediated by ATF4 (Fawcett *et al.*, 1999; Siu *et al.*, 2002). The examination of HepG2 cell lysates by immunoblotting shows that the accumulation of ATF4 protein occurs in the cells exposed to thapsigargin or arsenite (II, Fig. 4A). To find out whether ATF4 can bind to the 33-bp repeated sequence of the *hTRB3* promoter, we employed the electrophoretic mobility shift assay (EMSA) combined with the supershift analysis using ATF4-specific antibodies. The results reveal that exogenously expressed ATF4 is able to bind to the 33-bp repeat of *hTRB3* by forming two specific complexes (II, Fig. 4B). The complexes are abrogated by the mutation of the ATF as well as the C/EBP part of the C/EBP-ATF composite site, while the mutation of nucleotides flanking the C/EBP-ATF site retains ATF4 binding to the DNA probe. Thus, ATF4 can interact with the C/EBP-ATF site of the *hTRB3* promoter, and therefore, may participate in the transcriptional activation of the *hTRB3* gene. Indeed, the overexpression of ATF4 dramatically increases the expression of luciferase from the reporter constructs driven by the *hTRB3* promoter (II, Fig. 4D). Consistent with the results of EMSA, the mutations in the C/EBP-ATF site abolish the stimulation of luciferase expression by ATF4, but the mutations of the 5' or 3' part of the 33-bp repeat have no repressive effect (II, Fig. 4E). Thus, ectopically expressed ATF4 binds to and transactivates the *hTRB3* promoter *via* the C/EBP-ATF site without being affected by the nucleotide sequences flanking the C/EBP-ATF element within 33-bp repeat. To look whether the

endogenous ATF4 is involved in the activation of the *TRB3* gene, we over-expressed the dominant-negative ATF4 mutant in HepG2 cells. As a result, the *hTRB3* promoter-dependent expression of luciferase is inhibited by 90% or more in the cells exposed to thapsigargin or arsenite (II, Fig. 4C). Also, the downregulation of ATF4 by the expression of small interfering RNAs (siRNAs) directed against ATF4 decreases significantly the *hTRB3* promoter-driven reporter gene induction in the SaOS2 cells deprived of leucine (III, Fig. 5C). Thus, the endogenous ATF4 is involved in the stress-induced activation of the *hTRB3* promoter. In accordance with our results, the studies by Ohoka *et al*. (2005) demonstrate that exogenous ATF4 can activate the *hTRB3* promoter *via* C/EBP-ATF site and the siRNA-mediated knockdown of endogenous ATF4 in HEK293 cells abrogates the tunicamycin-induced *TRB3* expression. Also, the crucial role of ATF4 in the stress-dependent activation of *TRB3* has been recently confirmed by Jousse *et al*. (2007), who detected a strong *TRB3* mRNA accumulation in response to thapsigargin as well as to leucine starvation in wild-type MEFs, whereas no induction was observed in the ATF4-deficient cells. Collectively, the results described above indicate that ATF4 is able to bind and activate the C/EBP-ATF site of the *hTRB3* promoter and is critically involved in the stress-induced activation of *TRB3*.

There has been raised the question about the dimerization partner of ATF4 in the regulation of *TRB3* promoter. Since the C/EBP-ATF element is a composite of one-half of each of the palindromic sequences that comprise an C/EBP-binding motif and an ATF/CRE element, the binding partner for ATF4 is presumably a bZip transcription factor from the C/EBP subfamily. Confirming this assumption, the transcription factor C/EBPβ has been reported to be a partner of ATF4 for the transactivation of the C/EBP-ATF site in the *Chop* promoter (Ma *et al.*, 2002) and the NSRE-1 element in the *Asns* promoter (Siu *et al.*, 2002). Differently from these examples, Ohoka *et al*. (2005) showed that the expression of *TRB3* mRNA upon the ER stress is independent of the presence of C/EBPβ and another C/EBP subfamily member, CHOP, is instead implicated in the activation of the *TRB3* promoter. By the reporter assays carried out in this study, exogenous CHOP activates both two critical stress response elements in the *TRB3* promoter, the regulatory site in the 5' part of 33-bp repeat and the C/EBP-ATF composite site. Presumably, the activation of the latter one is mediated by CHOP heterodimerized with ATF4. However, the direct binding of CHOP to the 33-bp repeat of the *TRB3* gene is shown neither by EMSA nor chromatin immunoprecipitation, and the tunicamycin-triggered *TRB3* induction is only partially suppressed after the knockdown of the endogenous CHOP expression (Ohoka *et al.*, 2005). Therefore, while the ATF4 involvement in the transcriptional regulation of *TRB3 via* the C/EBP-ATF element has been established by now, the ATF4 interaction with CHOP in this process needs more experimental proofs.

3.6. TRB3 binds to ATF4 and inhibits its transcriptional activity (I, II)

To explore the functional role of the TRB3 protein and identify its potential interacting molecules, we employed the yeast two-hybrid analysis. The full-length mTRB3 was fused to the Gal4 DNA-binding domain and used as a bait to screen murine embryonic cDNA library fused to the VP16 activation domain. The interaction between TRB3 and its partner protein brings the Gal4 DNA-binding domain into close proximity of the VP16 activation domain, thereby producing the functional transcription activator complex VP16-Gal4. The complex drives the expression of a reporter gene, such as *HIS3* or *LacZ*, allowing the growth of cells in the medium lacking histidine, or producing a blue color in β-galactosidase staining assay, respectively. As a result of this selection procedure, we isolated 11 TRB3-specific positive clones, two of which encode the N-terminal fragments of ATF4. To map more precisely the TRB3-binding domain of ATF4, we made the deletion mutants of mATF4 (I, Fig. 4). The analysis of mutants by the yeast two-hybrid assay revealed that the TRB3 binding site resides between the amino acids 53 and 124 of mATF4, thus overlapping with the transcriptional activation domain of ATF4. In line with our results, yeast two-hybrid screening of the human liver cDNA library also detected ATF4 as the binding partner for TRB3 (Bowers *et al.*, 2003; Zhou *et al.*, 2008). Recently, Jousse *et al*. (2007) assessed the transcriptional activation activity of ATF4 mutants and found that the TRB3-binding region of ATF4 can be defined to residues 86-124.

We verified the formation of the TRB3-ATF4 complex in mammalian cells by the coimmunoprecipitation of the epitope-tagged mTRB3 and mATF4 (I, Fig. 6). Next, we aimed to investigate the intracellular distribution of TRB3. While the nuclear localization of ATF4 is well documented (Cibelli *et al.*, 1999), no information concerning the intracellular localization of TRB3 was available at that time. As predicted by PSORT II program (http://psort.nibb.ac.jp/), the N-terminus of mTRB3 contains two putative nuclear localization signals, RKKR (amino acids 15-18) and PVQKRAR (amino acids 30-36). To determine experimentally the subcellular localization of TRB3, we expressed mTRB3 fused to enhanced yellow fluorescence protein (EYFP) in cos-7, GT1-7 and CHO cells, and examined the cells in the fluorescence microscope. The experiment revealed that the mTRB3-EYFP fusion protein resides in the nuclei of cells, while EYFP by itself is distributed homogeneously throughout the cell (I, Fig. 3). Thus, mTRB3 contains a signal involved in the targeting of mTRB3-EYFP to the nuclei. The nuclear localization of TRB3 has been later repeatedly confirmed by other groups, and moreover, the presence of the nuclear localization signal in the N-terminus of TRB3 has also been proved (Wu *et al.*, 2003; Kiss-Toth *et al.*, 2005; Bezy *et al.*, 2007). Nevertheless, TRB3 has also been detected cytosolically near the plasma membrane, where TRB3 reportedly interacts with Akt kinase (He

et al., 2006), and with the components of the BMP signaling pathway, BMP type II receptor and Smurf1 (Chan *et al.*, 2007).

To elucidate the functional significance of the interaction between ATF4 and TRB3, we explored the possibility that TRB3 could affect the transactivation activity of ATF4. We conducted a series of reporter assays in CHO and cos-7 cells, using a chloramphenicol acetyltransferase gene driven by three tandem CRE sites (TGACGTCA) or by the E-selectin promoter containing single CRE. The results indicate that the coexpression of TRB3 suppresses dose-dependently the ATF4-activated transcription from the reporter constructs, and when approximately equal amounts of the expression constructs for ATF4 and TRB3 are transfected, the blockage of ATF4 activity by TRB3 is nearly total (I, Fig. 7).

As described in Chapter 3.5, *TRB3* is a target gene of ATF4. Therefore, we investigated whether TRB3 can suppress the induction of its own promoter. As expected, the ectopically expressed TRB3 almost completely suppresses the ATF4-dependent activation of the *hTRB3* promoter in the luciferase reporter assays (II, Fig. 5A). Moreover, TRB3 also inhibits the basal and thapsigargin- or arsenite-induced activity of the *hTRB3* promoter (II, Fig. 5A). Thus, the data indicate that TRB3 may function as a negative feedback regulator of ATF4. Recently, Jousse *et al*. (2007) have reached the same conclusion, reporting that overexpressed TRB3 significantly downregulates the expression of ATF4 target genes in leucine-starved HeLa cells.

To elucidate the molecular mechanism of the TRB3-dependent inhibition of ATF4 activity, we first asked whether mTRB3 promotes the degradation of ATF4, since Tribbles, the TRB3 ortholog in *Drosophila*, promotes the down-regulation of transcription factor Slbo through proteolytic degradation (Rorth *et al.*, 2000). However, as revealed by the pulse-chase labeling experiment in cos-7 cells, the degradation rate of ATF4 is not increased, but even slightly reduced in the cells coexpressing TRB3, compared to the cells expressing ATF4 alone (I, Fig. 8). In agreement with this, the steady-state level of ATF4 in transiently transfected cos-7 cells is elevated by the TRB3 coexpression. In accordance with our results, the exogenous TRB3 does not stimulate the degradation of endogenous ATF4 in leucine-depleted HeLa cells (Jousse *et al.*, 2007), but, in contrast, the coexpressed TRB3 enhances the proteolysis of ATF4 in U2-0S osteosarcoma cells (Bowers *et al.*, 2003). Thus, the effect of TRB3 on ATF4 degradation may depend on the cellular context, and at least in certain types of cells, mechanisms other than the stimulation of degradation are involved in the repression of ATF4 activity by TRB3. It is relevant to mention that the TRB3-dependent suppression of two other bZip transcription factors, CHOP and C/EBPβ, and PPARγ, the transcription factor from the nuclear receptor family, has been reported to occur without promoting their degradation (Ohoka *et al.*, 2005; Bezy *et al.*, 2007; Takahashi *et al.*, 2008).

To examine whether TRB3 interferes with the DNA binding activity of ATF4, we performed the EMSA with antibody supershift experiments. By using

the extracts from the cells (cos-7, HepG2) enriched with ectopically expressed ATF4 and TRB3, we detected that ATF4, in complex with TRB3, was able to bind to the CRE-containing oligonucleotide as well as to the 33-bp repeat of *hTRB3* containing C/EBP-ATF site (I, Fig. 9; II, Fig. 5B). In line with this, the recent chromatin immunoprecipitation experiments performed by Jousse *et al.* (2007) showed that TRB3 and ATF4 belong to the same protein complex bound to the *CHOP* AARE and *Asns* NSRE-1 site in leucine-deprived cells. Thus, TRB3 does not prevent ATF4 binding to its DNA elements. We have proposed that TRB3 suppresses ATF4 activity by interfering with the recruitment of the transcriptional apparatus, since the TRB3-binding site on ATF4 overlaps with the binding sites for transcriptional coactivators and general transcription factors (I, Fig. 4) (Liang and Hai, 1997). Indeed, as recently demonstrated, the overexpressed TRB3 can block the complex formation between ATF4 and the transcriptional coactivator p300 (Ohoka *et al.*, 2007). Similarly, TRB3 impedes the formation of the CHOP-p300 complex, which is supposedly the mechanism of inhibition of the CHOP transcriptional activity by TRB3 (Ohoka *et al.*, 2007). However, the possibility that TRB3 changes the posttranslational modification state of ATF4, which might lead to the inactivation of ATF4, is also worth to be investigated. As mentioned in Chapter 2.5, the phosphorylation of ATF4 modulates its transcriptional activity. It has been reported that TRB3 is able to abrogate the phosphorylation of critical regulatory sites on the NF-κB transactivator p65 (Wu *et al.*, 2003) and C/EBPβ (Bezy *et al.*, 2007) that causes the transcriptional inactivation of these factors.

In summary, TRB3 inhibits the transcriptional activity of ATF4 by binding to the N-terminal region of ATF4 that overlaps with the transcriptional activation domain. TRB3 does not prevent the binding of ATF4 to DNA or promote necessarily the degradation of ATF4.

3.7. TRB3 protects cells against the growth inhibitory and cytotoxic effect of ATF4 (III)

To investigate the roles of ATF4 and TRB3 in the regulation of cell growth and viability, we generated HEK293 cells stably transfected with tetracycline (tet)-inducible constructs, expressing, upon addition of tet to the culture medium, either ATF4 (ATF4-293 cells) or TRB3 (TRB3-293 cells) (III, Fig. 1A). By measuring the growth rate of tet-treated and untreated cells, we found that the enforced expression of ATF4 strongly suppresses the growth of cells, while the overexpression of TRB3 has no effect (III, Fig. 1C). Consistently, after plating at low densities, the colony-forming ability of tet-induced ATF4-293 cells is significantly decreased, compared to the tet-induced TRB3-293 cells and control cells (III, Fig. 1B). It has been reported previously that the over-expression of ATF4 in the mammary gland of transgenic mouse decreases

proliferation and increases apoptosis of the mammary epithelium (Bagheri-Yarmand *et al.*, 2003). Similarly to our result, the overexpressed TRB3 does not affect the proliferation of HeLa cells (Kiss-Toth *et al.*, 2005), but oppositely, TRB3 expression in lymphocytes induces the G2 cell cycle delay and cellular depletion (Selim *et al.*, 2007). Thus, similarly to the findings with *Drosophila* Tribbles (see Chapter 2.2), TRB3 appears to induce the cell cycle block in specific settings only. The mechanism underlying the TRB3-dependent block of mitosis in lymphocytes is currently unknown.

As described in the previous section, TRB3 interacts with ATF4 and inhibits its transcriptional activity. In order to study whether TRB3 is able to abrogate the cell growth inhibitory activity of ATF4, we introduced the tet-inducible TRB3 expression construct into ATF4-293 cells, resulting in HEK293 cells coexpressing ATF4 and TRB3 upon the addition of tet (the cells were named ATF4-TRB3-293) (III, Fig. 1E)., As expected, the transcriptional activity of ATF4 is markedly lower in these cells than in the control cells expressing ATF4 alone (ATF4-neo-293) (III, Fig. 1D). Importantly, the growth rate of the tet-treated ATF4-TRB3-293 cells is significantly increased in comparison to ATF4-neo-293 cells, reaching almost the level of tet-untreated cells (III, Fig. 1G). The colony growth assay of ATF4-TRB3-293 and ATF4-neo-293 cells confirms these results (III, Fig. 1F).

We also demonstrated that the coexpression of TRB3 rescues neuronally differentiated postmitotic PC12 cells from the apoptosis evoked by ATF4 overexpression (III, Fig. 2). Taken together, the foregoing results indicate that TRB3 is able to suppress the deleterious effects of ATF4 on the growth of proliferating cells as well as on the survival of postmitotic cells.

Since an essential function of ATF4 is to mediate cellular stress response, we investigated how the excessive expression of ATF4 affects the viability of glucose-deprived cells. This is a relevant stress situation to study, because glucose deficiency, a condition associated with several diseases like ischemia and tumors, upregulates both ATF4 (Siu *et al.*, 2002) and TRB3 (Schwarzer *et al.*, 2006). Our experiments showed that the HEK293 cells overexpressing ATF4 are extremely sensitive to the removal of glucose from the culture medium, resulting in about 80% cell death in 12 h, and the ATF4-dependent cell death is moderately delayed by the coexpression of TRB3 (III, Fig. 3A). The Northern blot analysis showed that the expression of ATF4 in the cells grown in the complete medium increases the level of *CHOP* and *GADD34* mRNA, and the expression of these genes is further augmented upon glucose deprivation from the culture medium (III, Fig. 4A). As reported previously by other researchers, the elevated expression of CHOP results in the exaggerated generation of reactive oxygen species (ROS) (McCullough *et al.*, 2001), and both CHOP and GADD34 promote the oxidation in the endoplasmic reticulum of stressed cells, leading to cell death (Marciniak *et al.*, 2004). To investigate whether the elevated expression of ATF4 increases the intracellular level of

ROS, we loaded the cells with the membrane-permeable dye 2',7'-dichlorodihydrofluorescein diacetate, which is ROS-dependently oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF), and measured the cellular fluorescence by flow cytometry. We detected a significant ROS generation in response to the elevated expression of ATF4 in ATF4-neo-293 cells (III, Fig. 4B), which is accompanied by a fall in the intracellular level of reduced glutathione (GSH) (III, Fig. 4C). The supplementation of the medium with reducing substances such as *N*-acetylcysteine or dithiothreitol corrects the growth defect of ATF4-overexpressing cells (III, Fig. 4D), and eliminates almost entirely the cytotoxicity of overexpressed ATF4 during glucose starvation (III, Fig. 4E). Importantly, the coexpression of TRB3 and ATF4 also results in the decrease of the intracellular ROS, accompanied by the elevated level of cellular GSH (III, Fig. 4A,B,C), showing that the ability of overexpressed ATF4 to generate ROS proceeds from its transcriptional activity, which is repressed by TRB3. Whether the ATF4-activated CHOP and/or GADD34 are the main sources of ATF4-dependent ROS generation remains to be investigated. Collectively, these results indicate that the growth inhibitory and cytotoxic effect of the elevated ATF4 in HEK293 cells is ROS-dependent, and TRB3, which downregulates ATF4 transcriptional activity, reduces the ATF4-dependent ROS generation.

In order to study whether the elevated expression of endogenous ATF4 is cytotoxic and endogenous TRB3 suppresses the effect of ATF4 in stressed cells, we used the silencing of *ATF4* and *TRB3* genes by RNAi in osteoblastic SaOS2 osteosarcoma cells. The leucine starvation is a condition that efficiently raises both the ATF4 and the TRB3 protein level in SaOS2 cells without increasing the death rate above 20% in 24 h treatment. The transfection of SaOS2 cells with siRNA directed against TRB3 or ATF4 significantly reduces the level of these proteins upon leucine starvation and, at the same time, increases or decreases, respectively, the transcriptional activity of endogenous ATF4 in the cells, as revealed by the results of luciferase reporter assays (III, Fig. 5A,B,C). SaOS2 cells preincubated in the leucine-free medium for 12 h are very sensitive to the following glucose starvation, resulting in the death rate of about 50% in 8 h treatment. While the downregulation of ATF4 by siRNA significantly lowers the death rate of the glucose-deprived cells that were preincubated in the leucine-free medium, the downregulation of TRB3 by siRNA has the opposite effect (III, Fig. 5D). The experiments with the SaOS2 cells which were exposed to glucose starvation for 22 h without preincubation in the leucine-free medium gave principally similar results (III, Fig. 5D). Thus, the silencing of *ATF4* and *TRB3* genes by RNAi revealed that ATF4 promotes and TRB3 suppresses the death of glucose-deprived SaOS2 cells. While it has been shown that the high expression level of ATF4 coincides with cell death in ischemic neurons suffering from the deprivation of oxygen and nutrients (Hayashi *et al.*, 2005; Li *et al.*, 2005), and the ectopic expression of TRB3 interferes with the apoptosis

of glucose-deprived PC-3 prostate carcinoma cells (Schwarzer *et al.*, 2006), our findings that the endogenous ATF4 acts in certain conditions as an anti-survival protein and endogenous TRB3 as a pro-survival protein have not been reported previously. In conclusion, the data presented in this work suggest that at the cellular level the biological role of TRB3, acting through the negative feedback mechanism, might be the protection of the cell from the excessive activity of ATF4. Furthermore, as demonstrated by Ohoka *et al*. (2005), TRB3 binds to and inhibits also the activity of CHOP which is the major proapoptotic target of ATF4 (see Chapter 2.6). However, Ohoka *et al*. (2005) have found that in certain conditions (the tunicamycin-induced ER stress) TRB3 sensitizes cells to apoptosis. The mechanism of TRB3-dependent cell death remains elusive and, as speculated, may be mediated not by the ATF4-CHOP pathway, but by some other regulator of cell viability. As described above, TRB3 has been implicated in the control of several signaling pathways that influence cell growth and survival, including Akt and MAPK pathways, the latter of which is linked to the ER stress-dependent cell death through the activation of JNK kinase (Du *et al.*, 2003; Kiss-Toth *et al.*, 2004; He *et al.*, 2006). Therefore, it seems likely that the role of TRB3 in the mechanism of cell death is affected by the crosstalk between the several signaling routes that may give different outcomes depending on the stress situation and cellular context.

4. CONCLUSIONS

During the course of investigation of the molecular basis of the TRB3 function and regulation we have characterized several aspects of its expression and potential role in stress response.

The most important conclusions of the thesis are the following:

1. The gene encoding pseudokinase TRB3 is upregulated in response to various cellular stresses, including nutrient deficiency, endoplasmic reticulum stress, and oxidative stress.
2. The stress-induced activation of *TRB3* gene is mediated by the promoter region containing C/EBP-ATF composite site(s).
3. ATF4 binds to the C/EBP-ATF composite site in the *TRB3* promoter and upregulates the expression of *TRB3* in response to stress.
4. TRB3 binds to and inhibits the transcriptional activity of ATF4, forming a negative feedback loop that regulates ATF4.
5. TRB3 protects cells against the growth inhibitory and cytotoxic effect of ATF4.

Our study reveals an important role for the stress-induced expression of TRB3 to serve as a component of the novel negative feedback loop used by the cell to control the activity of ATF4. By binding to ATF4, TRB3 directly represses ATF4 transcriptional activity, while GADD34, another stress-induced feedback inhibitor of ATF4, downregulates the translation of ATF4 *via* eIF2α dephosphorylation. The deregulated activity of ATF4 is associated with the diseases that involve stress-dependent cell death (ischaemia, diabetes, neuro-degenerative diseases), suggesting a possible role for TRB3 in these conditions. The characterization of the regulation of TRB3 expression and its role in stress response leads to a better understanding of mechanisms that control cell viability in physiological and pathophysiological conditions.

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SUMMARY IN ESTONIAN

Uurimus stressi poolt indutseeritavast pseudokinaasist TRB3, transkriptsioonifaktori ATF4 uuest inhibiitorist

Ebasoodsad muutused raku sise- ja väliskeskkonnas põhjustavad stressi, mille lahendamiseks käivitatakse rakus koordineeritud programm, stressivastus, mida juhitakse aktiveeritud signaaliradade abil. Stressivastus muudab geenide ekspressiooni nii transkriptsiooni kui ka translatsiooni tasemel ja aitab kohaneda olukorraga ning taastada homöostaasi. Juhul kui stress on väga intensiivne ja kohanemine pole võimalik, aktiveeritakse apoptoosiga seotud signaalirajad ja rakk elimineeritakse. Mitmete erisuguste rakuliste stresside korral (nagu aminohapete puudus, vale konformatsiooniga valkude kuhjumine endoplasmaatilises retiikulumis, nakatumine viirusega, heemipuudus, oksüdatiivne stress) kujundatakse ühine ehk integreeritud stressivastus (*integrated stress response*, ISR), kuna nimetatud stresside poolt aktiveeritavad signaalirajad konvergeeruvad ühes punktis, milleks on translatsiooni initsiatsioonifaktori eIF2α fosforüleerimine. eIF2α fosforüleerimine põhjustab valgusünteesi üldise pidurdumise, kuid samaaegselt soodustab teatud tüüpi mRNA-de transleerimist, millelt sünteesitakse stressivastuses osalevaid valke. Nii näiteks on soodustatud ISR-i käigus toimuva geenikspressiooni keskse regulaatori, aktiveeriva transkriptsioonifaktori 4 (ATF4) mRNA transleerimine. ATF4 on oluline rakkude homöostaasi taastamisel ja elulemusel, kuna ta aktiveerib suurt osa aminohapete metabolismiga seotud geenidest. Samas võib ATF4 olla seotud ka rakusurma programmi käivitamisega intensiivse stressi tingimustes, sest mõned ATF4 märklaudgeenidest on pro-apoptootilised (nagu transkriptsioonifaktor *CHOP*).

Käesolev töö sai alguse stressi poolt aktiveeritavate geenide otsingust hiire neuronaalses rakuliinis GT1-7, mille tulemusena meie töögrupp leidis seni kirjeldamata geeni [mis on praegu tuntud kui *Tribbles homolog 3* (*TRB3*) *alias NIPK*, *SKIP3*, *SINK*], mille mRNA tase tõuseb oluliselt närvirakkude surma põhjustavates tingimustes, näiteks kaltsiumi ainevahetuse häirete korral. Peagi publitseeriti mitmed artiklid meie poolt identifitseeritud valgule sarnasest äädikakärbse valgust Tribbles, mis blokeerib migreeruvate mesenhüümirakkude jagunemise gastrulatsiooni ajal. Struktuuri sarnasuse alusel on Tribbles valk ja tema kolm imetajates esinevat homoloogi – TRB1, TRB2 ja TRB3 – tänaseks ühendatud perekonda, mida tuntakse Tribbles-laadsete valkude nime all. Selle perekonna liikmed on kinaasitaolised valgud ehk pseudokinaasid, sest nad omavad seriin/treoniin-kinaasidele sarnast aminohappelist järjestust, milles puuduvad mõned valgukinaasides konserveerunud olulised motiivid, mistõttu nad tõenäoliselt ei oma kinaasset aktiivsust.

Käesoleva doktoritöö aluseks olevates teadusartiklites olen koostöös oma kaastöötajatega uurinud *TRB3* geeni transkriptsioonilist aktivatsiooni, TRB3

interaktsioone teiste valkudega ja TRB3 valgu rolli stressi all kannatavas rakus. Meie ja teiste rühmade tööde alusel on selgunud, et *TRB3* on stressitundlik geen – *TRB3* mRNA on indutseeritud mitmete erilaadsete stresside korral, nagu toitainete puudus, hüpoksia, arseniidi toksilisus ja endoplasmaatilise retiikulumi stress. Meie tööd selgitasid, et inimese *TRB3* mRNA-d on heterogeensed, erinedes 5' mittetransleeritavas osas (5'UTR), kusjuures stressi ajal suureneb selliste mRNA-de osakaal, mille 5'UTR moodustub varem kirjeldamata eksonist 1B või lühikestest ekson 1A variantidest. Kasutades konstrukte, milles reportergeeni ekspressioon sõltub inimese *TRB3* geeni 5' terminaalsetest fragmentidest, tuvastasime *TRB3* geeni indutseerumiseks vajaliku promootorpiirkonna ja tegime kindlaks, et regulatoorseks järjestuseks, mille vahendusel transkriptsiooni aktivatsioon stressi ajal toimub, on *TRB3* promootoris C/EBP-ATF liitelement. Edasised katsed näitasid, et ATF4 seondub C/EBP-ATF liitelemendile ja osaleb *TRB3* promootori aktiveerimisel. Kui tapsigargiini (endoplasmaatilise retiikulumi stressi tekitaja) või arseniidi stressi tingimustes ekspresseerida dominant-negatiivset ATF4 mutanti või leutsiinipuuduses olevates rakkudes vähendada endogeense *ATF4* geeni ekspressiooni RNA interferentsi (RNAi) abil, on *TRB3* promootoriga seotud reportergeeni induktsioon tugevasti (90% ja rohkem) inhibeeritud. Need tulemused viitavad ATF4 osalemisele *TRB3* geeni transkribeerimisel stressi tingimustes.

TRB3 rakusisese lokalisatsiooni määramisel fluorestsentsmikroskoopiliselt liitvalgu abil, mis koosnes TRB3-st ja modifitseeritud rohelisest fluorestsentsvalgust, selgus, et TRB3 suunab fluorestsentsvalgu rakutuuma. TRB3-ga interakteeruvate valkude otsing pärmi kaksikhübriidi meetodil näitas, et TRB3 seondub ATF4-ga, kusjuures TRB3 seondumisala paikneb ATF4 N-terminaalses osas, lõigus, mis hõlmab aminohappeid 53 kuni 124. Uurides TRB3 mõju ATF4 transkriptsioonilisele aktiivsusele ilmnes, et eksogeenne TRB3 inhibeerib ATF4 võimet stimuleerida reportergeeni ekspressiooni CRE või C/EBP-ATF järjestust sisaldavatelt promootoritelt. Ka tapsigargiini või arseniidi mõjul *TRB3* promootorilt indutseeritud reportergeeni ekspressioon on tugevasti pidurdatud eksogeense TRB3 poolt. Endogeense *TRB3* geeni ekspressiooni vähendamine RNAi abil tõstab ATF4 transkriptsioonilist aktiivsust leutsiinipuuduse korral. Seega näitavad käesolevas töös saadud tulemused, et TRB3 võib funktsioneerida ATF4 regulaatorina, mis töötab negatiivse tagasiside printsiibil. Täpne mehhanism, kuidas TRB3 inhibeerib ATF4 aktiivsust vajab veel selgitamist. Töös esitatud andmete järgi TRB3 ei kiirenda ATF4 valgu lagundamist ega takista ATF4 seondumist DNA-ga. Kuna TRB3 seondumiskoht on ATF4 transkriptsiooni aktivatsiooni domeenis, kuhu seonduvad ka üldised transkriptsioonifaktorid ja -aktivaatorid, on tõenäoline, et TRB3 takistab transkriptsiooni põhiaparaadi seondumist ATF4-ga.

Et selgitada TRB3 rolli stressi olukorras, valmistati HEK293 rakud, milles on TRB3 ja ATF4 ekspressiooni võimalik indutseerida tetratsükliini lisamisel söötmesse. Nende rakkude kasvukiiruse ja elulemuse mõõtmisel ilmnes, et

ATF4 ülemäärane ekspressioon aeglustab märkimisväärselt rakkude kasvu ja suurendab rakkude tundlikkust glükoosipuuduse suhtes. ATF4 toksilisus on seotud reaktiivsete hapnikuühendite (*reactive oxygen species*; ROS) taseme olulise tõusuga rakkudes ning on kõrvaldatav antioksüdantide (näiteks N-atsetüültsüsteiini) lisamisega söötmesse. ATF4 kasvupidurdavat ja tsütotoksilist toimet vähendab samuti koekspresseeritud TRB3, mis inhibeerib ATF4 aktiivsust ja hoiab ära ROS-ide taseme tõusu rakkudes. TRB3 koekspressioon kaitseb ka postmitootilisi, neuraalselt diferentseerunud PC12 rakke apoptoosi eest, mida põhjustab ATF4 kontrollimatu ekspressioon. Katsed, milles *ATF4* ja *TRB3* geenid vaigistati RNAi abil, kinnitavad, et endogeenne ATF4 suurendab ja TRB3 vähendab SaOS2 osteosarkoomi rakkude surma glükoosipuuduse tagajärjel. Saadud tulemused viitavad sellele, et TRB3 bioloogiliseks rolliks on raku kaitsmine ATF4 ülemäärase aktiivsuse eest. ATF4-ga seotud stressivastus on aktiveeritud paljude haiguste korral (nagu tuumorid, isheemia, diabeet, neurodegeneratiivsed haigused, luukoe väärarengud), mistõttu TRB3 võib etendada rolli nende haiguste tekkes ja kulgemises.

Kokkuvõttes on käesoleva töö peamised tulemused järgmised: TRB3 on ATF4 uus märklaudgeen, TRB3 seondub ATF4-ga ja inhibeerib ATF4 transkriptsioonilist aktiivsust. Nii meie kui ka teiste teaduslaborite töö tulemusena on tänaseks selgunud, et TRB3 üheks oluliseks funktsiooniks on rakusisese signalisatsiooni moduleerimise kaudu reguleerida rakkude stressivastust.

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PUBLICATIONS

I

Örd, D. and Örd, T. (2003)
Mouse NIPK interacts with ATF4 and affects its transcriptional activity.
Exp. Cell Res., 286, 308–320.

II

Örd, D. and Örd, T. (2005)
Characterization of human NIPK (TRB3, SKIP3) gene activation in stressful conditions. *Biochem. Biophys. Res. Commun.*, 330, 210–218.

III

Örd, D., Meerits, K. and Örd, T. (2007)
TRB3 protects cells against the growth inhibitory and cytotoxic effect of ATF4.
Exp. Cell Res., 313, 3556–3567.

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List of publications

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1997. a. kuni 1998. a. osalesin prof. Mart Ustavi töörühma projektis, milles uuriti veise papilloomiviiruse replikatsiooni mõjutavaid tegureid. Alates 1998 aastast töötan dr. Tõnis Ördi grupis. Peamiselt olen tegelenud eukarüootsete rakkude stressivastuse mehhanismide, geeniekspressiooni regulatsiooni ja valk-valk interaktsioonide uurimisega.

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Allikas, A., Örd, D., Kurg, R., Kivi, S., Ustav, M. (2001) Roles of the hinge region and the DNA binding domain of the bovine papillomavirus type 1 E2 protein in initiation of DNA replication. *Virus Res.*, 75, 95–106.

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