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Functions and regulation of the mammalian pseudokinase TRIB3

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Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Supervisors: Tõnis Örd, PhD, Senior Researcher
Estonian Biocentre
Tartu, Estonia

Jaanus Remme, PhD, Professor of Molecular Biology
University of Tartu
Tartu, Estonia

Opponent: Guillermo Velasco, PhD, Associate Professor
Complutense University of Madrid
Madrid, Spain

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LIST OF ORIGINAL PUBLICATIONS

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

I **Örd, T.**, Örd, D., Kõivomägi, M., Juhkam, K. and Örd, T. (2009). Human TRB3 is upregulated in stressed cells by the induction of translationally efficient mRNA containing a truncated 5′-UTR. *Gene* 444: 24–32.

II **Örd, T.**, Örd, D., Kuuse, S., Plaas, M. and Örd, T. (2012). *Trib3* is regulated by IL-3 and affects bone marrow-derived mast cell survival and function. *Cell Immunol* 280: 68–75.

III **Örd, T.**, Innos, J., Lilleväli, K., Tekko, T., Sütt, S., Örd, D., Kõks, S., Vasar, E. and Örd, T. (2014). *Trib3* is developmentally and nutritionally regulated in the brain but is dispensable for spatial memory, fear conditioning and sensing of amino acid-imbalanced diet. *PLoS One* 9: e94691.

IV **Örd, T.**, Örd, D., Adler, P., Vilo, J. and Örd, T. (2015). TRIB3 enhances cell viability during glucose deprivation in HEK293-derived cells by upregulating IGFBP2, a novel nutrient deficiency survival factor. *Biochim Biophys Acta* 1853: 2492–2505.

My contributions to the articles are as follows:

Ref. I conceived, validated and applied the real-time PCR-based assay system for *TRIB3* mRNA isoform quantification, conducted a portion of the cell culture work, constructed a subset of the plasmids, and participated in data analysis, the writing of the manuscript and the preparation of the figures,

Ref. II contributed to the study design and data interpretation, performed most of the *in vitro* mast cell sensitization and activation treatments and degranulation measurements, conducted all RT-qPCR experiments, participated in the generation and cultivation of BMMCs and in the validation and genotyping of *Trib3* knockout mice, was involved in carrying out the *in vivo* passive cutaneous anaphylaxis experiment, performed part of the statistical analysis and figure preparation, wrote the manuscript,

Ref. III contributed to the study design and data interpretation, carried out the behavioral experiments and dietary treatments, performed the RT-qPCR work (except for brain region dissection and the preparation of the developmental cDNA time series), analyzed lateral

ventricles from location-matched sections, performed Southern blotting and other additional *Trib3* knockout mouse validation experiments, conducted the statistical analyses, prepared the figures (except for brain section imaging and annotation) and wrote the manuscript,

Ref. IV contributed to the study design and data interpretation, carried out the genome-wide gene expression analysis (with P. Adler), gene set functional profiling and transcription factor motif enrichment analysis, conducted a major portion of the cell culture work (including a major subset of the transfections, treatments and viability analyses), performed the RT-qPCR work, measured protein synthesis by radiolabeling, participated in Western blotting, carried out the imaging of cell morphology and the construction of plasmids, conducted the statistical analyses, prepared the figures and wrote the manuscript.

ABBREVIATIONS

ACC	acetyl-CoA carboxylase
Akt	murine (Ak strain) thymoma viral oncogene homolog
AP-1	activator protein 1
APC	anterior piriform cortex
APC/C	anaphase-promoting complex/cyclosome
APOBEC3A	apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3A
ATF	activating transcription factor
β-TrCP	β-transducin repeat-containing E3 ubiquitin protein ligase
BCL	B-cell lymphoma proto-oncogene
BiP	immunoglobulin heavy chain-binding protein
BMMC	bone marrow-derived mast cell
BMP	bone morphogenetic protein
BMPR2	BMP type II receptor
bZIP	basic region–leucine zipper
C/EBP	CCAAT/enhancer-binding protein
Cas9	CRISPR-associated protein 9
CASK	Ca^{2+}/calmodulin-activated serine/threonine kinase
caspase	aspartate-directed cysteine protease
CDC	cell division cycle
CDH1	CDC20 homolog 1
CHOP	C/EBP homologous protein
COP1	constitutive photomorphogenesis protein 1 homolog
CREB	cAMP response element-binding protein
CRISPR	clustered regularly-interspaced short palindromic repeats
CtIP	C-terminal-binding protein-interacting protein
DDIT3	DNA damage-inducible transcript 3
E	embryonic day
E1A	adenovirus early region 1A
E2F	factor activating adenovirus E2 promoter
E3	third enzyme of the ubiquitin transfer cascade
EAA	essential amino acid
eIF	eukaryotic translation initiation factor
ELAVL1	embryonic lethal, abnormal vision-like RNA-binding protein 1
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Fos	Finkel-Biskis-Jinkins murine osteosarcoma virus oncogene homolog
FoxO	forkhead box, subgroup O
FZR1	fizzy/CDC20-related 1
GADD34	growth arrest and DNA damage-inducible protein 34
GCN2	general control non-derepressible 2
GM-CSF	granulocyte-macrophage colony-stimulating factor

GRP78	glucose-regulated protein, 78 kDa
HER3	human epidermal growth factor receptor 3
HRI	heme-regulated inhibitor
HSPA5	heat shock protein family A (HSP70) member 5
HuR	Hu antigen R
IgE	immunoglobulin E
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IL	interleukin
INHBE	inhibin βE
IRS	insulin receptor substrate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
Jun	avian sarcoma virus 17 oncogene homolog (‘ju-nana’, Japanese for the number 17)
KAT2B	lysine acetyltransferase 2B
LC3	microtubule-associated protein 1 light chain 3α
Maf	avian musculoaponeurotic fibrosarcoma virus oncogene homolog
MAPK(K)(K)	mitogen-activated protein kinase (kinase) (kinase)
MEK1	MAPK/ERK kinase 1
MELK	maternal embryonic leucine zipper kinase
MKK7	MAPKK 7
MLK3	mixed lineage kinase 3
mTOR	mechanistic target of rapamycin
mTORC	mTOR complex
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NF-κB	nuclear factor-κB
NIPK	neuronal cell death-inducible putative kinase
NRSN2	neurensin 2
ORF	open reading frame
P	postnatal day
PARK2	Parkinson’s protein 2, E3 ubiquitin protein ligase
PCAF	p300/CREB-binding protein-associated factor
PDGF-BB	platelet-derived growth factor, formed from two B chains
PERK	PKR-like endoplasmic reticulum kinase
PEST	proline, glutamic acid, serine and threonine-enriched
PI3K	phosphoinositide 3-kinase
PKR	protein kinase R
PPAR	peroxisome proliferator-activated receptor
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
Ras	rat sarcoma retroviral oncogene homolog
RBBP8	retinoblastoma-binding protein 8

RelA	avian reticuloendotheliosis viral oncogene homolog A
RFWD2	RING (Really Interesting New Gene) finger and WD repeat domain 2
Rho	Ras homolog gene
RICTOR	rapamycin-insensitive companion of mTOR
RT-qPCR	reverse transcription-qPCR
SCF	S-phase kinase-associated protein 1, Cullin, F-box protein
SgK495	Sugen kinase 495
SHIK	SINK-homologous serine/threonine protein kinase
SIAH1	seven in absentia homolog family E3 ubiquitin protein ligase 1
SINK	p65-interacting inhibitor of NF-κB
SKIP3	stress kinase inhibitory protein 3
Slbo	slow border cells
SMAD	sma and mothers against decapentaplegic homolog
SMURF	SMAD-specific E3 ubiquitin protein ligase
SMYD1	SET and MYND domain-containing 1
SQSTM1	sequestosome 1
STAT	signal transducer and activator of transcription
STK40	serine/threonine kinase 40
Tet	tetracycline
TGF	transforming growth factor
TNF	tumor necrosis factor
TRIB	tribbles homolog
uORF	upstream ORF
UTR	untranslated region
WNK	with no lysine
XBP1	X-box binding protein 1

1. INTRODUCTION

Tribbles homolog 3 (*TRIB3*; also known as *TRB3*, *NIPK*, *SKIP3* and *SINK*) is a mammalian gene that is strongly activated in response to several forms of cellular stress, *i.e.*, environmental conditions that are unfavorable to the cell. Stressors that induce *TRIB3* expression in diverse cell types include nutrient deficiencies, oxidative stress, hypoxia and endoplasmic reticulum (ER) stress—conditions that may occur to a certain degree in normal physiology but which also contribute to the pathology of several common diseases, including cancer, stroke and myocardial infarction.

The protein encoded by *TRIB3* contains a single distinct domain, which displays sequence similarity to the protein kinase domain. However, several amino acid residues which are highly conserved in catalytically active protein kinases, and are considered to be important for the functioning of these enzymes, have been substituted in the TRIB3 kinase-like domain. Due to this, TRIB3 is classified as a pseudokinase and significant phosphotransferase activity is considered unlikely. As such, TRIB3 functions by forming protein–protein interactions and thereby regulating the activity of other proteins, including several transcription factors, protein kinases and ubiquitin ligases. Through such interactions, TRIB3 participates in the regulation of the cellular stress response and has the potential to affect cell survival. Additionally, TRIB3 is also implicated in developmental processes, inflammation and metabolism. The known mechanisms of *TRIB3* regulation and function are the focus of the literature overview presented in this dissertation.

Through the work done previously by our group and others, *TRIB3* has emerged as an exciting gene with the potential to participate in multiple facets of mammalian biology. For the experimental part of this dissertation, several novel aspects of the role and regulation of the *TRIB3* gene were studied at the cell and organism levels, facilitated by the generation of a *Trib3* knockout mouse line by our group.

To acquire a better understanding of *TRIB3* upregulation in response to stress, comparative quantification of different *TRIB3* mRNA isoforms was performed in human hepatoma cells, revealing that the mRNA population composition shifts markedly in stressed cells. Studying the role of mouse *Trib3* in *in vitro*-differentiated mast cells, a type of tissue-resident immune cell, uncovered a growth factor-sensitive manner of *Trib3* expression in these cells as well as links between *Trib3* and cellular immunological responses. In the brain, cellular stress response pathways are known to mediate cognitive processes as part of normal physiology, which prompted the analysis of *Trib3* expression in the developing and adult mouse brain and the characterization of the behavioral phenotype of mice that lack *Trib3*. Finally, the role of *TRIB3* in the cellular stress response to glucose deficiency was investigated in cell culture, identifying TRIB3-modulated genes using genome-wide gene expression profiling and leading to new insight into the regulation of cell survival in nutrient-poor conditions.

Taken together, the results signify that TRIB3, a single-domain protein with impaired catalytic activity, performs a complex of roles, many of which are likely to be cell type-specific.

2. REVIEW OF LITERATURE

2.1. The discovery of *Drosophila* Tribbles and mammalian TRIB3

In the world of science fiction, tribbles first appeared on December 29, 1967, when the *Star Trek: The Original Series* episode entitled "The Trouble with Tribbles" was originally aired in the United States (Okuda and Okuda, 1997). Central to the plot of this episode are tribbles, a species of alien organisms that are depicted as small, round, furry and soft, and are thus adored by the crew of the spaceship and introduced onboard as pets. However, as tribbles proliferate unbelievably quickly and prove hard to contain, they soon threaten to consume all the supplies available on the ship. Eventually, tribbles manage to enter the cargo hold, which is filled with grain that the ship is on a mission to transport, and proceed to consume the cargo. Unexpectedly, when the crew discovers that the grain has been consumed, they also notice that the tribbles are dying as a result of this, revealing to the crew that the cargo they had been transporting was poisoned all along.

Tribbles were linked to the world of biology in the year 2000, when several investigators reported on a previously uncharacterized *Drosophila melanogaster* (fruit fly) gene (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Rorth *et al.*, 2000; Seher and Leptin, 2000). In mutant embryos deficient of this gene, a profound over-proliferation of mesodermal cells was evident during the gastrulation phase, and it was this observation that led Seher and Leptin to name the novel gene *tribbles*, after the uncontrollably proliferating alien species from the *Star Trek* television series (Seher and Leptin, 2000). In *Drosophila*, Tribbles, the protein encoded by the *tribbles* gene, inhibits mitosis during early embryonic development by binding to and inducing the ubiquitination of the phosphatase String, an ortholog of mammalian cell division cycle 25 (CDC25), subsequently leading to proteasomal degradation of String and cell cycle arrest in the G2 phase (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000).

In mammals, there are three genes that are considered to encode homologs of *Drosophila* Tribbles (*TRIB1*, *TRIB2* and *TRIB3*), and their discoveries actually precede that of the fruit fly *tribbles* gene which they were eventually named after. In 1996, *TRIB2* (originally designated *c5fw*) was isolated from a thyroid cDNA library, followed by *TRIB1* (originally designated *c8fw*) in 1997, also from thyroid tissue (Wilkin *et al.*, 1996; Wilkin *et al.*, 1997). *TRIB3* was first described as neuronal cell death-inducible putative kinase (*NIPK*), a gene that was strongly upregulated in rat neuronal cells during programmed cell death brought on by nerve growth factor deprivation or treatment with the calcium ionophore A23187 (Mayumi-Matsuda *et al.*, 1999). Independently, the mouse gene corresponding to *TRIB3* was first isolated by our group, using differential cloning to search for genes that are upregulated in neuronal GT1-7 cells during apoptotic cell death induced by treatment with thapsigargin, an inhibitor of ATP-dependent calcium uptake into the ER (Örd and Örd, 2003).

At approximately the same time, the human *TRIB3* gene was described by several groups who designated it either *SKIP3* (stress kinase inhibitory protein 3), *SINK* (p65-interacting inhibitor of NF-κB) or *TRB3* (tribbles homolog 3) (Bowers *et al.*, 2003; Du *et al.*, 2003; Kiss-Toth *et al.*, 2004; Wu *et al.*, 2003).

Recently, the organization responsible for approving human gene names, the HUGO Gene Nomenclature Committee, has redefined the meaning of TRIB3 to "tribbles pseudokinase 3", instead of its previously approved definition, "tribbles homolog 3 (Drosophila)" (data available at: www.genenames.org, accessed 2016-01-29). On the other hand, the organization that provides official names for mouse genes, the Mouse Genomic Nomenclature Committee, currently continues to define *Trib3*, the mouse ortholog of human *TRIB3*, as "tribbles homolog 3 (Drosophila)" (data available at: www.informatics.jax.org/mgihome/nomen, accessed 2016-01-29). According to the official nomenclatures for human and mouse genes (referenced above), the proteins that are produced from mouse *Trib3* and human *TRIB3* are both referred to as TRIB3.

2.2. The structure of the TRIB3 protein

2.2.1. The peculiar kinase-like domain of Tribbles family proteins

The characteristic feature of *Drosophila* as well as vertebrate Tribbles proteins is a central domain that possesses sequence similarity to the serine/threonine protein kinase domain but contains several amino acid substitutions at catalytically important positions, leading to the classification of Tribbles proteins as pseudokinases (Boudeau *et al.*, 2006; Hegedus *et al.*, 2006; Hegedus *et al.*, 2007). The kinase-like domain, which is approximately 250–300 amino acid residues in length, comprises the majority of the protein, and it is flanked by less-conserved, short (approximately 40–80 amino acid residues long) N- and C-terminal regions (Figure 1) (Hegedus *et al.*, 2006; Hegedus *et al.*, 2007). Human and mouse TRIB3 proteins, at 358 and 354 amino acid residues in length, respectively, correspond to the typical Tribbles family protein structure, and their predicted molecular weights are approximately 40 and 39 kDa, respectively (Bowers *et al.*, 2003; Örd and Örd, 2003).

2.2.1.1. The state of catalytic motifs in the Tribbles pseudokinase domain

Close to ten percent of the more than 500 protein kinases encoded in the human genome are considered pseudokinases due to the lack of one or more conserved amino acid motifs that are critical for the catalytic activity of 'classical' protein kinase domains (Boudeau *et al.*, 2006; Manning *et al.*, 2002). Pseudokinases exist among many different protein kinase subfamilies and different pseudokinases feature distinct sets of alterations to the canonical catalytically important residues (Boudeau *et al.*, 2006). In the region corresponding to the ATP binding site in classical protein kinases, Tribbles proteins lack a canonical

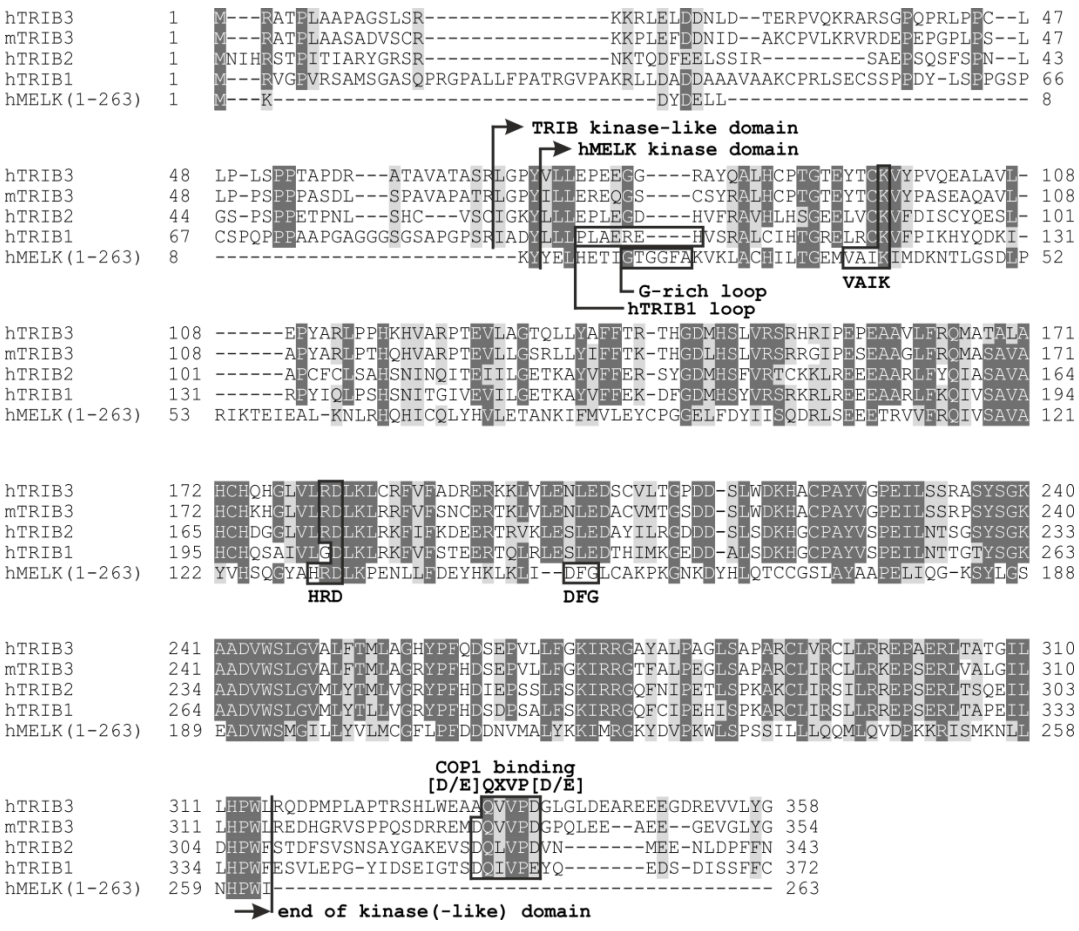

Figure 1. Comparison of the amino acid sequences of the three human (h) Tribbles homolog pseudokinases (hTRIB3, hTRIB2 and hTRIB1), the mouse (m) ortholog of hTRIB3, and hMELK, the most hTRIB3-similar human kinase with canonical catalytic motifs and robust protein kinase activity. Of the typical protein kinase domain motifs, the putative ATP binding region of TRIB proteins lacks a canonical glycine-rich loop but does contain the conserved lysine residue in the VAIK motif. In the catalytic loop (the HRD motif), the catalytic aspartic acid residue is present but the histidine residue has been substituted. The DFG motif, which binds Mg^{2+} ions in canonical kinases, is completely absent in TRIB proteins. For hTRIB1, crystal structure analysis has revealed a loop with the sequence PLAEREH in place of the glycine-rich loop of canonical kinases (3D structures for other TRIB proteins are not available). Outside of the kinase-like domain, a motif with the amino acid sequence [D/E]QXVP[D/E] can be identified in the C-terminal region of TRIB proteins; this motif has been found to mediate binding to the E3 ubiquitin ligase COP1. For references, see main text. The protein sequences were obtained from UniProt entries Q96RU7 (hTRIB3), Q8K4K2 (mTRIB3), Q92519 (hTRIB2), Q96RU8 (hTRIB1) and Q14680 (hMELK) (data available at: www.uniprot.org, accessed 2016-01-29; UniProt Consortium, 2015). For hMELK, only the region extending from the N-terminus to the end of the kinase domain is included (amino acid residues 1–263). The extent of the protein kinase-(like) domains was determined based on PROSITE (available at: prosite.expasy.org, accessed 2016-01-29, database version 20.122; Sigrist *et al.*, 2013). The sequences were aligned using the T-Coffee multiple sequence alignment tool (available at: www.ebi.ac.uk/tools/msa/tcoffee, accessed 2016-02-01, version 8.93, BLOSUM matrix; Notredame *et al.*, 2000). The alignment was visualized with BioEdit (version 7.0.9.0; Hall, 1999). COP1, constitutive photomorphogenesis protein 1 homolog; MELK, maternal embryonic leucine zipper kinase.

glycine-rich loop, while the highly conserved lysine residue in the VAIK motif is generally present (Boudeau *et al.*, 2006; Hanks and Hunter, 1995; Hegedus *et al.*, 2007). In the region that corresponds to the catalytic loop, the catalytic aspartic acid residue from the HRD motif is conserved in Tribbles proteins; however, the nearly-invariant histidine residue in the catalytic core HRD motif is not conserved (Hanks and Hunter, 1995; Hegedus *et al.*, 2006). Finally, Tribbles proteins lack the conserved DFG motif that is required for binding Mg^{2+} in canonical protein kinases (Hanks and Hunter, 1995; Hegedus *et al.*, 2007).

2.2.1.2. Scrutinizing the (lack of) kinase activity in Tribbles proteins

Due to the striking deviations from canonical protein kinase structure (described in the previous section), Tribbles proteins have been predicted to be catalytically inactive, and, in agreement with this, *in vitro* experiments designed to detect the phosphorylation of classical non-specific kinase substrate proteins by TRIB3 or TRIB2 have been unable to demonstrate phosphotransferase activity (Bowers *et al.*, 2003; Wilkin *et al.*, 1997).

In light of this, a recent report by Bailey *et al.* (2015) showing that human TRIB3 and TRIB2 possess the ability to auto-phosphorylate *in vitro*, albeit very weakly, is of considerable interest. Such a revelation is not without precedent among pseudokinases, as there have been several cases where proteins with kinase-like domains were initially predicted to lack kinase activity, due to missing catalytic motifs, but were subsequently shown to be functional protein kinases (examples include WNK1, CASK and HER3) (Boudeau *et al.*, 2006; Mukherjee *et al.*, 2008; Shi *et al.*, 2010; Xu *et al.*, 2000). In these cases, the phosphorylation reaction often proceeds by non-standard catalytic mechanisms. For example, in the WNK ('with no lysine') kinases, the VAIK motif of the ATP binding pocket lacks the canonical lysine residue, and a lysine residue from a different strand is found in the active site (Min *et al.*, 2004; Xu *et al.*, 2000). Similarly to the Tribbles proteins, the protein kinase CASK lacks the canonical metal-binding aspartic acid residue in the DFG motif; however, CASK was eventually found to function as a Mg^{2+}-independent kinase, demonstrating that Mg^{2+} binding is not indispensable for kinase activity (Mukherjee *et al.*, 2008).

For TRIB3 and TRIB2, it was shown that the ability to auto-phosphorylate requires the conserved lysine residue of the VAIK motif in the ATP binding site, and that this auto-phosphorylation only occurs in the absence of divalent metal cations (Bailey *et al.*, 2015). Further experiments with TRIB2 confirmed that it binds ATP with an affinity that could be physiologically relevant, and, in line with the auto-phosphorylation results, the binding of ATP occurs in a manner dependent on the conserved lysine residue of the ATP binding pocket (Bailey *et al.*, 2015). In contrast, human TRIB1 was found to be unable to bind ATP *in vitro*, irrespective of the presence or absence of Mg^{2+} (Murphy *et al.*,

2015). Therefore, it is possible that the family-wide systematic presence and conservation of unusual kinase domain motif variants represents enzymatic adaptations of Tribbles proteins, but the enzymatic capabilities probably do not manifest in all family members. As of now, the significance of TRIB3 and TRIB2 kinase activity remains uncertain, as only a very low level of phosphotransferase activity has been demonstrated under particular *in vitro* conditions, and the biological function of this activity has not been elucidated, nor have any cellular substrates been identified (Bailey *et al.*, 2015).

2.2.1.3. Insights into Tribbles pseudokinase domain geometry from the first 3D structure

In late 2015, the crystal structure of human TRIB1 (without its N-terminal region) was published, representing the first experimental three-dimensional structure available for any member of the Tribbles family (Murphy *et al.*, 2015). In general, the structure of a protein kinase domain is made up of an N-terminal lobe that comprises the binding site for ATP, and a C-terminal lobe that is considered to be mainly responsible for binding the peptide substrate (Hanks and Hunter, 1995). In the structure of the TRIB1 kinase-like domain, the C-terminal lobe was found to adopt a conformation akin to that of canonical kinases, while the structure of the N-terminal lobe deviated considerably from that of conventional kinases, resulting in a deformed ATP binding pocket that appears to preclude nucleotide binding (Murphy *et al.*, 2015). Thus, the TRIB1 active site geometry is in agreement with the lack of ATP binding ability observed experimentally for TRIB1 (Murphy *et al.*, 2015). Since TRIB2 and TRIB3 present (weak) ATP binding/auto-phosphorylation abilities, differences in their amino acid sequences compared to TRIB1 presumably lead to structural rearrangements in the ATP binding region, substituting or relocating occluding residues (Bailey *et al.*, 2015; Eyers, 2015; Murphy *et al.*, 2015).

2.2.2. The N- and C-terminal regions of Tribbles family proteins

Outside of the kinase-like domain, the N-terminal segment in Tribbles family proteins is characterized by high serine and proline content, and could serve as a PEST (proline, glutamic acid, serine and threonine-enriched) region, an element that reduces the intracellular half-life of a protein by promoting its degradation (Hegedus *et al.*, 2007). The half-life of the TRIB3 protein can indeed be very short in certain conditions, even as short as 15 minutes (Ohoka *et al.*, 2010) or a few hours (Humphrey *et al.*, 2010; Zhou *et al.*, 2008b), due to the degradation of TRIB3 by the ubiquitin–proteasome system. However, in different conditions, the half-life of TRIB3 protein has been determined to be approximately 10 hours or more (Liew *et al.*, 2010; Wennemers *et al.*, 2012); thus, the turnover rate of TRIB3 protein is strongly context-dependent.

Additionally, the N-terminal regions of TRIB3 and TRIB1 contain a functional nuclear localization signal, while the TRIB2 protein contains no apparent nuclear localization signal (Kiss-Toth *et al.*, 2006). In line with this, TRIB3 and TRIB1 are readily imported into the nucleus (Kiss-Toth *et al.*, 2006; Örd and Örd, 2003; Wu *et al.*, 2003; Yokoyama *et al.*, 2010), while TRIB2 is generally not nuclear (Kiss-Toth *et al.*, 2006; Wang *et al.*, 2013a; Wilkin *et al.*, 1997), although the presence of TRIB2 in the nucleus has also been reported (Wang *et al.*, 2013b). Although TRIB3 is often predominantly localized to the nucleus, major roles for TRIB3 (discussed subsequently) have been reported in both the nucleus and the cytoplasm.

The C-terminal segments of the Tribbles family proteins contain two conserved motifs (Hegedus *et al.*, 2006; Hegedus *et al.*, 2007; Yokoyama and Nakamura, 2011), and the functional significance of these motifs has been elucidated. The motif with the consensus sequence ILLHPWF is the binding site for MEK1, a mitogen-activated protein kinase kinase (MAPKK) (Yokoyama *et al.*, 2010; Yokoyama and Nakamura, 2011), while the motif [D/E]QXVP[D/E] serves as the binding site for the E3 ubiquitin ligase COP1, which is recruited by Tribbles proteins to mediate proteasomal destruction of target proteins (Keeshan *et al.*, 2010; Qi *et al.*, 2006).

2.2.3. How distinct is TRIB3 in terms of protein sequence?

Beyond the short N- and C-terminal motifs described above, sequence conservation amongst the Tribbles family, and between the Tribbles family proteins and other proteins, is mostly limited to the kinase-like domain (Hegedus *et al.*, 2006). The sequence of the kinase-like domain of human TRIB3 is 55% identical to that of human TRIB2, 54% identical with human TRIB1, and 45% identical to the kinase-like domain of *Drosophila* Tribbles (Örd, 2008), indicating considerable intra-family divergence. Notably, TRIB1 and TRIB2 are substantially more similar to each other (71% sequence identity in humans) than to TRIB3 (comparisons mentioned above) (Yokoyama and Nakamura, 2011). Comparing the kinase-like domains of Tribbles homologs in human and mouse, TRIB3 appears to be relatively less conserved than the other Tribbles family members, with murine TRIB3 demonstrating 81% sequence identity to human TRIB3, while for TRIB1 and TRIB2 the level of sequence identity between human and mouse is greater than 97% (Yokoyama and Nakamura, 2011). Less stringent between-species conservation of TRIB3, compared to TRIB1 and TRIB2, appears to be a general trend among mammals, as indicated by the phylogenetic tree of Tribbles-like sequences generated by Hegedus *et al.* (2006).

Excluding the other Tribbles homologs, the human protein most similar to human TRIB3 is STK40 (‘serine/threonine kinase 40’, also known as SgK495 and SHIK), which has a kinase-like domain with 37% sequence identity to that of TRIB3, and is also a pseudokinase (Bailey *et al.*, 2015; Boudeau *et al.*, 2006;

Huang *et al.*, 2003). Curiously, the set of canonical catalytic motifs that are either missing or present in the STK40 kinase-like domain is similar to that of the Tribbles proteins; however, the kinase-like domain of STK40 is considered severely degraded due to insertions and substitutions, and STK40 has failed to show auto-phosphorylation ability *in vitro* (Bailey et al., 2015).

Following STK40, the next most similar human kinases compared to TRIB3 are several kinases from the calcium/calmodulin-dependent protein kinase family. Among these, the human TRIB3 kinase-like domain has the highest degree of identity, 30%, with the kinase domain of maternal embryonic leucine zipper kinase (MELK), a catalytically active kinase with canonical catalytic motifs (Gil *et al.*, 1997). Several crystal structures of human MELK have recently been made available in the Protein Data Bank (the earliest structure being PDB ID: 4BL1, contributed by Canevari *et al.*, unpublished in literature as of 2016-01-12). Thus, MELK is also the most TRIB3-similar active kinase that has a three-dimensional structure available.

2.2.4. Post-translational modifications of TRIB3

A number of post-translational modifications of the TRIB3 protein are known to occur (summarized in Table 1), acting alongside other mechanisms to regulate *TRIB3* gene output.

According to the PhosphoSitePlus database (available at: www.phosphosite.org, accessed 2015-10-19; Hornbeck *et al.*, 2012), a total of five phosphorylation sites (four in human, one in mouse) have been observed for TRIB3 so far. However, each of these modification sites has only been reported in a one study, and all of these studies are high-throughput proteomic analyses using mass spectrometry (Hornbeck *et al.*, 2012; Mertins *et al.*, 2013; Rinschen *et al.*, 2010; Zhou *et al.*, 2013b). Furthermore, no kinases have been associated with these putative phosphorylation events, nor has functional significance been assigned to these modifications. As described in section 2.2.1.2, TRIB3 demonstrates a low level of auto-phosphorylation *in vitro*. However, the modification site(s) and the functional importance of this activity have not been elucidated (Bailey *et al.*, 2015). Taken together, the phosphorylation of TRIB3 probably does occur *in vivo*, but the details of this modification are currently uncertain.

Other post-translational modifications of TRIB3 include ubiquitination (Ohoka *et al.*, 2010; Zhou *et al.*, 2008b), methylation (Rasmussen *et al.*, 2015) and acetylation (Yao and Nyomba, 2008). The ubiquitination of TRIB3 leads to the proteasome-mediated degradation of TRIB3 and has been reported to take place *via* a direct protein–protein interaction with the E3 ubiquitin ligase SIAH1 (Zhou *et al.*, 2008b) as well as by physical interaction with CDH1 and CDC20, co-activators of the E3 ubiquitin ligase APC/C (‘anaphase-promoting complex/cyclosome’) (Ohoka *et al.*, 2010). TRIB3 is lysine-methylated by SMYD1, a histone methyltransferase for which TRIB3 is a non-histone

substrate (Rasmussen *et al.*, 2015). Acetylation of TRIB3 has been reported in the rat liver, and prenatal alcohol exposure leads to the hypo-acetylation of TRIB3 (Yao and Nyomba, 2008).

A post-translational truncation of human TRIB3 occurs when it is cleaved by caspases at position D338, resulting in the elimination of a C-terminal fragment that is twenty amino acid residues in length (Shimizu *et al.*, 2012). Several caspases (caspase-3, -6, -7, -8, -9 and -10), have been shown to perform the cleavage of TRIB3 at D338, and they utilize only this site to cleave TRIB3 (additionally, caspase-2 cleaves at D343 and/or D351) (Shimizu *et al.*, 2012). The caspase-mediated cleavage of TRIB3 has been proposed to regulate the activation of apoptotic processes (Shimizu *et al.*, 2012), as will be discussed subsequently.

Table 1. Post-translational modifications that have been described for TRIB3.

Modification	Result of modification	Reference(s)
Acetylation	• Reduced TRIB3 acetylation is associated with insulin resistance	Yao and Nyomba (2008)
Methylation	• Potentiation of SMYD1-mediated repression of transcription	Rasmussen *et al.* (2015)
Phosphorylation	• Significance currently unknown	Bailey *et al.* (2015)
Proteolytic cleavage	• Caspase-mediated site-specific truncation of TRIB3 protein at the C-terminal end; regulation of apoptosis	Shimizu *et al.* (2012)
Ubiquitination	• Proteasomal degradation of TRIB3 protein	Ohoka *et al.* (2010); Zhou *et al.* (2008b)

2.3. The regulation of *TRIB3* gene expression

2.3.1. The tissue distribution of *TRIB3* mRNA expression

In a healthy adult mouse, *Trib3* mRNA is expressed in a variety of tissues. A high level of expression is consistently detected in the liver, and substantial levels of expression have also been reported (with some inconsistencies between studies) in the small intestine, stomach, heart, kidney, lung, skin, brown adipose tissue, white adipose tissue, cerebellum, salivary gland, bone, pancreas, placenta and eye, while the level of *Trib3* mRNA expression tends be low in skeletal muscle, forebrain, spleen, thymus, ovary, uterus and testis (Lattin *et al.*, 2008; Okamoto *et al.*, 2007; Örd and Örd, 2003; Pontius *et al.*, 2003; Wu *et al.*, 2009). Comparing the tissue expression patterns of the three Tribbles homologs present in the mouse, there is some overlap between the

genes as well as some apparent differences. For instance, all three Tribbles homologs are expressed in the mouse heart, kidney, lung, adipose tissue and skin (Okamoto *et al.*, 2007). The liver, a major site of *Trib3* expression, is also a major site of *Trib1* (but not *Trib2*) expression (Okamoto *et al.*, 2007). On the other hand, the small intestine and stomach are relatively major sites of *Trib3* expression, but this is not the case for *Trib1* or *Trib2* (Okamoto et al., 2007).

In line with the results from mouse, human multi-tissue mRNA panels consistently confirm that *TRIB3* expression is high in the liver, compared to many other tissues (Bowers *et al.*, 2003; Wu *et al.*, 2003). In broad terms, the tissue expression profile of human *TRIB3* resembles that of mouse *Trib3*, although there are some notable differences that appear across multiple studies, such as relatively low expression in the human heart and high expression in the human spleen and thymus (Bowers *et al.*, 2003; Kiss-Toth *et al.*, 2004; Pontius *et al.*, 2003; Uhlen *et al.*, 2015; Wu *et al.*, 2003). Additionally, human tissue profiling also highlights the thyroid gland, bone marrow and peripheral blood leukocytes as sites of high *TRIB3* mRNA expression (Kiss-Toth *et al.*, 2004; Uhlen *et al.*, 2015).

It would also be of interest to compare the abundance of TRIB3 protein across different tissues or cells; unfortunately, such data is scarce. The Human Protein Atlas project has performed immunohistochemical analysis of TRIB3 using an extensive panel of tissues; however, the reliability of this data is categorized as 'uncertain', since their in-house antibody validation procedure revealed issues with the anti-TRIB3 antibody that was used (product name: HPA015272, data available at: www.proteinatlas.org, accessed 2015-10-19; Uhlen *et al.*, 2015). Problems with the specificity and sensitivity of antibodies that are available against TRIB3 have also been documented by others (for example, Wennemers *et al.*, 2011b).

2.3.2. *TRIB3* expression is induced in response to diverse types of cellular stress

A major feature of *TRIB3* gene expression is the upregulation of the *TRIB3* mRNA level in response to diverse types of cellular stress. For example, *TRIB3* is induced in cells suffering from ER stress (the accumulation of unfolded proteins in the ER lumen), oxidative stress (an excess of reactive oxygen species) or hypoxia (inadequate oxygen supply) (Bowers *et al.*, 2003; Lange *et al.*, 2008; Mayumi-Matsuda *et al.*, 1999; Morse *et al.*, 2010; Ohoka *et al.*, 2005; Örd and Örd, 2003; Wennemers *et al.*, 2011a). Essential amino acid deficiency, which impairs protein synthesis, is also known to lead to the upregulation of *TRIB3* (Carraro *et al.*, 2010; Jousse *et al.*, 2007; Örd *et al.*, 2007). Other nutritional stressors that activate *TRIB3* include free fatty acids (including saturated fatty acids, *e.g.*, palmitic acid, and polyunsaturated fatty acids, *e.g.*, docosahexaenoic acid) and glucose deficiency as well as excess (Geng *et al.*, 2013; Liu *et al.*, 2010; Morse *et al.*, 2010; Qian *et al.*, 2008; Schwarzer *et al.*, 2006; Shaikh *et al.*, 2008; Slagsvold *et al.*, 2010; Yacoub Wasef *et al.*, 2006).

Various chemical toxicants, such as arsenite, carbon tetrachloride and cadmium, also increase the level of *TRIB3* mRNA expression (Campos *et al.*, 2014; Hsiao and Stapleton, 2009; Örd and Örd, 2005; Permenter *et al.*, 2011; Wu *et al.*, 2003), as do ionizing radiation (Kim *et al.*, 2014a) and viral infection (Liao *et al.*, 2013; Yamane *et al.*, 2009). Extracellular signals, such as the pro-inflammatory cytokine tumor necrosis factor (TNF) or the depletion of nerve growth factor, and even mechanical stretch, can also elevate *TRIB3* expression (Cheng *et al.*, 2015; Mayumi-Matsuda *et al.*, 1999; Wu *et al.*, 2003).

Furthermore, *TRIB3* is induced in response to several drugs that are currently approved or being investigated as anti-cancer therapeutics, for example, the proteasome inhibitor bortezomib, the multikinase inhibitor sorafenib, the nuclear factor-κB (NF-κB) inhibitor DHMEQ, the cannabinoid tetrahydro-cannabinol, the nonsteroidal anti-inflammatory drug celecoxib, the peroxisome proliferator-activated receptor α/γ (PPARα/γ) activator ABTL0812, and salinomycin, a novel anti-cancer compound with an uncertain mechanism of action (Cervello *et al.*, 2013; Cusimano *et al.*, 2010; Erazo *et al.*, 2015; Lampiasi *et al.*, 2009; Li *et al.*, 2013; Locatelli *et al.*, 2013; Milani *et al.*, 2009; Salazar *et al.*, 2009b; Vara *et al.*, 2011). Notably, genotoxic stress (induced either by the DNA intercalating agent doxorubicin or the topoisomerase inhibitor etoposide) does not appear to increase *TRIB3* expression (Corcoran *et al.*, 2005; Sakai *et al.*, 2010).

Taken together, there is an extensive body of literature showing that the level of *TRIB3* mRNA expression is subject to upregulation in a wide range of mechanistically distinct stress conditions.

2.3.3. The basis of cellular stress-induced *TRIB3* gene activation

2.3.3.1. The *TRIB3* gene contains a C/EBP–ATF composite site

The human *TRIB3* gene is located in the short arm of chromosome 20, at bands p13–p12.2, and spans approximately 17 kbp (NCBI Gene ID: 57761). The human *TRIB3* and mouse *Trib3* (NCBI Gene ID: 228775) genes have a similar structure. The first exon encompasses the entire 5′-untranslated region (5′-UTR) of the mRNA and thus the very first nucleotides of the second exon constitute the translation initiation codon. The second and third exons are entirely protein-coding, and the protein-coding region, which is approximately 1100 nt long in total, ends in the fourth and final exon. The stop codon is followed by a 3′-UTR which is close to 1 kb in length.

Several regulatory elements have been identified in the *TRIB3* gene. The transcription regulation element which mediates the induction of *TRIB3* in response to many types of cellular stress is termed a C/EBP–ATF composite site, and it is located approximately one hundred base pairs upstream of frequently used transcription start sites in the human *TRIB3* gene (Ohoka *et al.*, 2005; Örd and Örd, 2005). In addition to human *TRIB3*, the C/EBP–ATF site has been determined to be conserved and functional in the mouse *Trib3* gene as well (Carraro *et al.*, 2010).

C/EBP–ATF composite sites are binding sites for basic region–leucine zipper (bZIP) transcription factor heterodimers consisting of a CCAAT/enhancer-binding protein (C/EBP) family transcription factor and an activating transcription factor (ATF) family transcription factor, and the consensus sequence of C/EBP–ATF sites (5′-NTTNCATCA-3′) is, accordingly, composed of half-sites for each family (Kilberg *et al.*, 2009). During cellular stress, C/EBP–ATF sites typically serve as positive (transcription-activating) regulatory elements, and they satisfy the criteria for classification as enhancer elements, since they function in an orientation- and position-independent manner (Han *et al.*, 2013; Kilberg *et al.*, 2009; Palii *et al.*, 2004; Zhong *et al.*, 2003).

Intriguingly, the C/EBP–ATF element of the human *TRIB3* gene is located on a 33-bp tandem repeat, with the regulatory element situated approximately in the center of the repeating sequence (Ohoka *et al.*, 2005; Örd and Örd, 2005). The copy number of this repeat is polymorphic in the human population, with a range of 2–5 copies, and all of the four reported alleles appear to be quite prevalent (Liew *et al.*, 2010; Örd and Örd, 2005). In a reporter assay using a heterologous promoter construct containing either three or six copies of the *TRIB3* 33-bp repeat, the doubling of the repeat copy number entailed an approximately two-fold increase in reporter gene activity in both basal and cellular stress conditions (Chaveroux *et al.*, 2015), indicating that the copy number of the repeat may be positively associated with the level of *TRIB3* expression. However, it is currently unexplored whether the natural copy number variation of the *TRIB3* 33-bp repeat affects *TRIB3* levels in the human population.

2.3.3.2. ATF4 and the 'integrated stress response': driving transcription from C/EBP–ATF sites as a result of eIF2α phosphorylation

The transcription factor ATF4 serves as the master regulator of transcription induction from C/EBP–ATF response elements, and, during cellular stress, ATF4 directly binds to and activates the expression of at least around two hundred genes *via* these elements (Han *et al.*, 2013; Shan *et al.*, 2009).

ATF4 is the central transcriptional regulator of the integrated stress response pathway, wherein ATF4 is induced by a translational mechanism in response to the phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α) at serine 51 (Harding *et al.*, 2003; Lu *et al.*, 2004). In mammalian cells, the phosphorylation of eIF2α may be carried out by four kinases (GCN2, PERK, PKR and HRI), which are activated in response to distinct indications of cellular stress, such as amino acid deficiency, the accumulation of unfolded proteins in the ER, viral infection and oxidative damage (Galabru *et al.*, 1989; Harding *et al.*, 1999; Lu *et al.*, 2001; Meurs *et al.*, 1990; Sood *et al.*, 2000; Wek *et al.*, 2006). Thus, the responses to diverse types of cellular stress converge on the

induction of ATF4, which in turn activates transcription using C/EBP–ATF response elements. ATF4 target genes include genes that function to adapt the cells to the stress conditions, genes that are further regulators of the stress response, and, in cases of prolonged and irresolvable stress, genes that induce cell death (Han *et al.*, 2013; Harding *et al.*, 2003; Wek *et al.*, 2006).

2.3.3.3. The composition of C/EBP and ATF transcription factors at the *TRIB3* promoter during stressful conditions

It has been demonstrated that ATF4 is able to bind to the *TRIB3* C/EBP–ATF composite site and thereby activate *TRIB3* gene expression in response to several types of stress (Ohoka *et al.*, 2005; Örd and Örd, 2005). Additional transcription factors that bind to the same DNA element in the *TRIB3* gene have also been identified, and some of them probably represent heterodimerization partners for ATF4, notably C/EBP homologous protein (CHOP), C/EBPβ and C/EBPγ. An overview of the transcriptions factors reported to directly regulate *TRIB3* gene expression is presented in Table 2.

CHOP, a stress-inducible member of the C/EBP transcription factor family, is able cooperate with ATF4 to promote *TRIB3* induction in response to cellular stress, and possibly binds to the C/EBP–ATF site as a heterodimer with ATF4 (Ohoka *et al.*, 2005). Additionally, C/EBPβ, C/EBPγ and ATF2 have also been shown to bind to the proximal promoter region of *TRIB3* (Carraro *et al.*, 2010; Huggins *et al.*, 2015; Selim *et al.*, 2007). As with CHOP, ATF4 can also heterodimerize with C/EBPβ and C/EBPγ, and these heterodimers also bind to C/EBP–ATF composite sites (Huggins *et al.*, 2015; Lopez *et al.*, 2007; Mann *et al.*, 2013; Podust *et al.*, 2001).

C/EBPβ and C/EBPγ recruitment has been shown to increase mouse *Trib3* expression (Huggins *et al.*, 2015; Li *et al.*, 2008; Morse *et al.*, 2010; Selim *et al.*, 2007). On the other hand, ATF2 binding to the mouse *Trib3* proximal promoter region does not appear to be altered by stress, and the deletion of the *Atf2* gene does not appear to affect the level of *Trib3* expression (Carraro *et al.*, 2010). This is in line with results obtained studying other genes, where it has been detailed that not all transcription factors bound to a functional C/EBP–ATF response element affect transcription driven by that element (Averous *et al.*, 2004). Note that at least in rodents, the *Trib3* proximal promoter region contains an additional possible binding site for the C/EBP family members. In mouse and rat, a CHOP–C/EBP composite site (consensus sequence: 5′-[G/A][G/A][G/A]TGCAAT[A/C]CCC-3′) has been identified in close proximity to the *Trib3* C/EBP–ATF site, and mutating either site decreases promoter activity in a reporter assay (Cheng *et al.*, 2015; Selim *et al.*, 2007; Ubeda *et al.*, 1996).

Thus, the landscape of transcription factor dimers that may be recruited to the *TRIB3* C/EBP–ATF (and possibly CHOP–C/EBP) site(s) is certainly complex; however, when viewed from the endpoint of achieving *TRIB3* mRNA induction in response to stress, the identified transcriptional regulators are

actually quite interdependent. *CHOP* is known to be a direct ATF4 target gene that is strongly induced by ATF4 (Fawcett *et al.*, 1999; Ma *et al.*, 2002). Similarly, the expression levels of *C/EBPβ* and *C/EBPγ* are also highly ATF4-dependent (Harding *et al.*, 2003; Huggins *et al.*, 2015). Furthermore, from a chromatin structure standpoint, it has been reported that ATF4 functions as a 'pioneer factor' for C/EBP–ATF site-dependent transcription activation, facilitating histone acetylation in the target locus to trigger an open chromatin state (Shan *et al.*, 2012). Taken together, the induction of ATF4 may be considered the critical and initial step in the C/EBP–ATF site-mediated upregulation of *TRIB3* during stressful conditions.

2.3.4. Additional direct regulators of *TRIB3* transcription

In addition to the C/EBP and ATF family members, the transcription factors PPARα, FoxO1 and NFATc1 have been reported to directly regulate *TRIB3* transcription (Table 2) (Chan *et al.*, 2011; Koo *et al.*, 2004; Matsumoto *et al.*, 2006). PPARα, a regulator of hepatic fatty acid metabolism, augments *TRIB3* expression in liver cells, though the promoter element mediating this effect has only been localized to a 400-bp region (Koo *et al.*, 2004). FoxO1 binds to the murine *Trib3* promoter region and has been reported to either activate or repress *Trib3* expression (in neuronal cells and hepatocytes, respectively), and several putative binding sites that correspond to the consensus FoxO response element have been proposed (Matsumoto *et al.*, 2006; Zareen *et al.*, 2013). NFATc1 is capable of transcriptionally activating *TRIB3* in vascular smooth muscle cells treated with phenamil (an ion channel inhibitor), and an NFAT consensus sequence mediating this effect has been identified at approximately 1 kbp upstream of the *TRIB3* transcription initiation sites (Chan *et al.*, 2011).

2.3.5. Human *TRIB3* mRNA isoforms

The human *TRIB3* mRNA population is heterogeneous, consisting of several mRNA isoforms that differ in their 5′-UTR (*i.e.*, the first exon) as a result of alternative transcription initiation sites and alternative splicing (Örd and Örd, 2005). Two alternative variants of the first exon have been uncovered, designated 1A and 1B, which arise from different transcription initiation regions (Örd and Örd, 2005). While the 1A variant utilizes a single splice donor site, at least four alternative splice donor sites (designated 1B1, 1B2, 1B3 and 1B4) have been demonstrated for exon 1B, allowing for a total of at least five different mRNA 5′-UTR splice isoforms (Örd and Örd, 2005). Additional complexity of the *TRIB3* mRNA pool arises from alternative transcription initiation sites: more than ten transcription start sites, spanning a region of more than 300 bp, have been discovered for splice variant 1A, and close to ten mRNA synthesis start sites, which span a region of approximately 50 bp, have been identified for splice variant 1B (Örd and Örd, 2005).

Table 2. An overview of different modes of *TRIB3* gene expression regulation. Only regulators that are reported to act directly upon the *TRIB3* gene or mRNA are included in this table.

Regulator	Result of regulation	Reference(s)
Transcriptional regulation		
ATF4	• Activation of *TRIB3* transcription in response to several types of cellular stress, including ER stress, oxidative stress and nutrient deprivation	Ohoka *et al.* (2005); Örd and Örd (2005); Örd *et al.* (2007)
C/EBPβ	• Activation of *TRIB3* transcription in response to cellular stress, such as oxidative stress	Morse *et al.* (2010); Selim *et al.* (2007)
C/EBPγ	• Activation of *TRIB3* transcription in response to cellular stress, such as amino acid deprivation	Huggins *et al.* (2015)
CHOP (DDIT3)	• Activation of TRIB3 transcription in response to several types of cellular stress, such as ER stress and oxidative stress	Morse *et al.* (2010); Ohoka *et al.* (2005)
FoxO1	• Activation of *TRIB3* transcription in response nerve growth factor deprivation in neuronal cells • Repression of *TRIB3* transcription in hepatocytes	Matsumoto *et al.* (2006); Zareen *et al.* (2013)
NFATc1	• Activation of *TRIB3* transcription in response to phenamil, an ion channel inhibitor	Chan *et al.* (2011)
PPARα	• Activation of *TRIB3* transcription in response to PPARα ligand	Koo *et al.* (2004)
Post-transcriptional regulation		
HuR (ELAVL1)	• Elevation of *TRIB3* mRNA level in response to anoxia	Rzymski *et al.* (2008)
miR-24	• Reduction of *TRIB3* mRNA level in response to platelet-derived growth factor-BB (PDGF-BB)	Chan *et al.* (2010)
miR-96	• Reduction of *TRIB3* mRNA level in the absence of bone morphogenetic protein 4 (BMP4)	Kim *et al.* (2014b)

Since all the known exon 1 variants join exon 2 using the same splice acceptor site, the *TRIB3* open reading frame (ORF) is identical in the different mRNA isoforms, and mRNA sequence variation is limited to the (entire) 5′-UTR (Örd and Örd, 2005). Variation of the 5′-UTR may have a major influence on the rate of protein production, as mRNA structural elements located in the 5′-UTR control the efficiency of translation initiation, a critical point of regulation for eukaryotic translation (Kozak, 2005). Mammalian mRNAs may have highly GC-rich 5′-UTRs, which can reduce translation by as

much as 100-fold in some cases (Han *et al.*, 2003), and other 5′-UTR sequence elements that can have a similar magnitude of effect on translation efficiency are known as well (Ghilardi *et al.*, 1998; Kozak, 2001; Kozak, 2005; Lee *et al.*, 1999).

2.3.6. Post-transcriptional regulation of *TRIB3*

The *TRIB3* transcript has a rapid turnover rate, with mRNA half-life measurement results ranging from slightly less than 20 minutes (Rzymski *et al.*, 2008) to approximately 2 or 3 hours (Selim *et al.*, 2007; Sharova *et al.*, 2009; Yacoub Wasef *et al.*, 2006). In the comprehensive study by Sharova *et al.* (2009), where the mRNA half-life for nearly 20,000 genes was measured in mouse embryonic stem cells, only 9% of genes had a shorter mRNA half-life than *Trib3* (half-life 2.8 hours), and, in the cell type investigated, only a total of 54 genes (0.3%) showed mRNA half-lives less than 1 hour, while nearly 1,800 genes (9%) showed an mRNA half-life of more than 20 hours. The median mRNA half-life for all genes was determined to be 7.1 hours in mouse embryonic stem cells (Sharova *et al.*, 2009), which is comparable to that reported for hepatoma cells (a median of 10 hours for a sample of approximately 5,200 genes; Yang *et al.*, 2003).

Some mechanisms that affect the degradation rate of the *TRIB3* transcript have been identified (Table 2). Under anoxic conditions, the RNA-binding protein HuR has been implicated in stabilizing human *TRIB3* mRNA (Rzymski *et al.*, 2008), and human *TRIB3* mRNA is also a direct target of at least two microRNAs, miR-24 and miR-96, through microRNA recognition elements located in the mRNA 3′-UTR (Chan *et al.*, 2010; Hsu *et al.*, 2014; Kim *et al.*, 2014b).

2.4. The functions of TRIB3

While TRIB3 has been found to possess a slight level of catalytic activity under specific *in vitro* conditions, it is not currently known to carry out any biological role as a kinase. Rather, TRIB3 is known to function by forming protein–protein interactions with a variety of different binding partners, including several transcription factors (Table 3), protein kinases (Table 4), ubiquitin ligases (Table 5) and other proteins (Table 6). In terms of subcellular localization, TRIB3 apparently functions in the nucleus and the cytoplasm. The interaction of another protein with TRIB3 may result in TRIB3 blocking functionally important regions of the interactor, resulting in the inhibition of its activity. In other cases, TRIB3 may act as an adaptor or scaffolding protein, targeting one protein to the proximity of another. Using these modes of action, TRIB3 participates in a range of intracellular signaling processes, typically acting as a negative regulator.

2.4.1. TRIB3 regulates the cellular stress response and cell death

As described in section 2.3.2, a major distinctive feature of *TRIB3* gene expression is its induction in response to cellular stress. In keeping with this, TRIB3 has been found to physically interact with and regulate several factors central to the cellular stress response and cell death. Intriguingly, depending on the cellular context and stress type, TRIB3 possesses substantial pro- as well as anti-survival roles in stressed cells. At the same time, genome-wide loss-of-function screens utilizing CRISPR/Cas9 or gene trap methods in haploid human cell lines, which enables the direct generation of pools of knockout cells, have revealed that TRIB3 is non-essential for cell proliferation and survival under standard culture conditions (TRIB1 and TRIB2 likewise; Blomen *et al.*, 2015; Wang *et al.*, 2015). This is compatible with a stress- or other stimulus condition-dependent and/or cell type-specific role for *TRIB3*.

2.4.1.1. TRIB3 is a negative feedback inhibitor of ATF4, a master regulator of stress response transcription

One of the known functions of TRIB3 in the context of cellular stress is the inhibition ATF4 (Bowers *et al.*, 2003; Örd and Örd, 2003), a bZIP transcription factor that is, as mentioned previously (2.3.3.2), the central transcriptional activator of the integrated stress response to diverse types of cellular stress. Since *TRIB3* is among the genes activated by ATF4 in response to stress (2.3.3.3), the inhibition of ATF4 by TRIB3 provides a negative feedback mechanism for the regulation of ATF4 activity (Ohoka *et al.*, 2005; Örd and Örd, 2005).

Early on, *Drosophila* Tribbles was found to negatively regulate target proteins, including the bZIP transcription factor Slbo, by promoting their proteasome-mediated destruction (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Rorth *et al.*, 2000; Seher and Leptin, 2000). However, TRIB3 is able to inhibit ATF4 without increasing the rate of ATF4 degradation (Örd and Örd, 2003). Rather, TRIB3 appears to function by directly repressing the transcriptional activity of ATF4 (Ohoka *et al.*, 2005; Örd and Örd, 2003; Örd and Örd, 2005).

TRIB3 binds to the transactivation domain of ATF4 and blocks the association between ATF4 and its transcriptional co-activators, such as the histone acetyltransferase p300 (Ohoka *et al.*, 2007; Örd and Örd, 2003). When in complex with TRIB3, ATF4 is still able to bind its target DNA sequence, thus it is possible that the interaction between ATF4 and TRIB3 takes place while ATF4 is bound to its target genes (Örd and Örd, 2003).

The modulation of ATF4 activity by TRIB3 has been found to be important for the fine-tuning of ATF4-dependent transcription in stressed cells, and, during severe stress where the ATF4-driven transcriptional program promotes cell death, endogenous TRIB3 is able to prolong cell survival by restricting endogenous ATF4 activity (Jousse *et al.*, 2007; Örd *et al.*, 2007).

Table 3. Transcription factors reported to physically interact with TRIB3. Only interactions with known functional significance are included in this table.

Protein	Result of interaction	Biological significance	Reference(s)
ATF4	Inhibition of ATF4 transcriptional activity	• Negative feedback regulation of ATF4 activity • Regulation of the cellular stress response • Suppression of cell death	Bowers *et al.* (2003); Jousse *et al.* (2007); Ohoka *et al.* (2005); Örd and Örd (2003); Örd and Örd (2005); Örd *et al.* (2007)
C/EBPβ	Inhibition of C/EBPβ transcriptional activity	• Inhibition of adipocyte differentiation • Potential for negative feedback regulation of C/EBPβ activity	Bezy *et al.* (2007); Selim *et al.* (2007)
CHOP (DDIT3)	Inhibition of CHOP transcriptional activity	• Negative feedback regulation of CHOP activity	Ohoka *et al.* (2005); Ohoka *et al.* (2007)
PPARγ	Inhibition of PPARγ transcriptional activity	• Inhibition of adipocyte differentiation	Takahashi *et al.* (2008)
RelA (p65)	Inhibition of NF-κB transcriptional activity	• Negative feedback regulation of NF-κB activity • Inhibition of pro-inflammatory responses • Promotion of cell death	Duggan *et al.* (2010); Smith *et al.* (2011); Wu *et al.* (2003)
SMAD3	Potentiation of SMAD3 transcriptional activity; maintenance of SMAD3 nuclear localization	• Positive feedback regulation of TGF-β–SMAD3 activity • Promotion of tumor cell migration and invasion	Hua *et al.* (2011)

2.4.1.2. TRIB3 inhibits CHOP and C/EBPβ, bZIP transcription factors situated downstream of ATF4

CHOP and C/EBPβ are C/EBP family transcription factors that form heterodimers with ATF4 for the regulation of C/EBP–ATF composite site-dependent transcription (Fawcett *et al.*, 1999; Lopez *et al.*, 2007; Mann *et al.*, 2013; Ohoka *et al.*, 2005; Podust *et al.*, 2001), and both CHOP and C/EBPβ have a relationship with TRIB3 that is reminiscent of the relationship between ATF4 and

TRIB3. That is, TRIB3 is able to physically interact with and inhibit the transcriptional activity of CHOP as well as C/EBPβ, and, since both of these transcription factors can also activate the *TRIB3* promoter, there exists the potential for TRIB3 to provide negative feedback regulation of CHOP and C/EBPβ activity in conditions of cellular stress (Bezy *et al.*, 2007; Carraro *et al.*, 2010; Li *et al.*, 2008; Ohoka *et al.*, 2005; Ohoka *et al.*, 2007; Selim *et al.*, 2007).

Given that both CHOP and C/EBPβ are inducible by ATF4 during stress (Fawcett *et al.*, 1999; Harding *et al.*, 2003; Ma *et al.*, 2002), TRIB3 has the capability to provide negative feedback of multiple successive steps of the stress-associated transcriptional program. In the transcriptional network controlling the cellular stress response, CHOP is regarded as a decidedly pro-apoptotic transcription factor, especially in the context of ER stress, and multiple apoptosis-promoting mechanisms that act downstream of CHOP have been identified (Li *et al.*, 2014). Likewise, C/EBPβ can also assert a negative effect on cell viability by facilitating apoptosis in response to challenging conditions (Meir *et al.*, 2010; Mukherjee *et al.*, 2001; Sun *et al.*, 2005; Zinszner *et al.*, 1998). Therefore, the inhibition of CHOP- and C/EBPβ-dependent pro-apoptotic transcription by TRIB3 represents a potential mechanism for augmenting cell survival.

Consequently, it is intriguing that there are exist combinations of stress and cell type in which the ATF4–CHOP pathway induces *TRIB3* expression, and cell death is triggered in a CHOP-dependent manner, yet the ultimate effect of TRIB3 on cell survival is pro-death, rather than a pro-survival effect as would be expected for a feedback inhibitor of the pro-apoptotic CHOP (Ohoka *et al.*, 2005; Shang *et al.*, 2009; Shang *et al.*, 2010). Apparently, TRIB3 can alternatively act as a downstream 'death executor' for CHOP; a potential mechanism for this is described in the next section.

2.4.1.3. Inhibition of Akt by TRIB3 in the context of cellular stress

In the liver, TRIB3 was discovered to be an inhibitor of the protein kinase Akt, specifically its isoforms Akt1 and Akt2, in the context of insulin signaling (Du *et al.*, 2003), though this function of TRIB3 has been called into question, at least under a normal metabolic state (Iynedjian, 2005; Okamoto *et al.*, 2007). However, in cells suffering from stress, Akt can exert a pro-survival effect, and the inhibition of Akt activity is a way by which stress-induced TRIB3 can provoke cell death (Borsting *et al.*, 2014; Cheng *et al.*, 2015; Humphrey *et al.*, 2010; Humphrey *et al.*, 2014; Salazar *et al.*, 2013; Zareen *et al.*, 2013).

Mechanistically, TRIB3 was found to directly bind to Akt1/2 and reduce the phosphorylation of Akt at serine 473 and threonine 308, two modifications that indicate Akt activation, and to reduce the phosphorylation of Akt target proteins (Du *et al.*, 2003). The total amount of Akt protein does not appear to be affected by TRIB3 (Du *et al.*, 2003). The phosphorylation of Akt at serine 473 is carried out by the mechanistic target of rapamycin complex 2 (mTORC2), and recently

TRIB3 was shown to interact with the kinase mTOR as well as with RICTOR, the mTORC2-specific companion of mTOR (Borsting *et al.*, 2014). Based on this, it has been suggested that the binding of mTORC2 by TRIB3 may also be involved in the mechanism of TRIB3-mediated Akt inhibition (Borsting *et al.*, 2014). The inhibitory effect of TRIB3 on Akt activation is enhanced by the MAPK kinase kinase MLK3, which forms a complex with TRIB3 and stabilizes the TRIB3 protein, leading to increased Akt inhibition and reduced cell survival (Humphrey *et al.*, 2010).

Table 4. Protein kinases reported to physically interact with TRIB3. Only interactions with known functional significance are included in this table.

Protein	Result of interaction	Biological significance	Reference(s)
Akt1/2	Inhibition of Akt activation	• Reduction of Akt kinase activity • Suppression of tumorigenesis • Promotion of apoptosis • Reported to affect as well as not affect insulin signaling, glucose metabolism and insulin resistance	Du *et al.* (2003); Humphrey *et al.* (2010); Iynedjian (2005); Koh *et al.* (2013); Okamoto *et al.* (2007); Prudente *et al.* (2005); Salazar *et al.* (2015); Zou *et al.* (2009)
BMPR2	BMP4-sensitive inhibition of SMURF1 degradation	• Positive regulation of BMP and TGF-β signaling • Facilitation of osteoblastic differentiation • Maintenance of smooth muscle cell phenotype	Chan *et al.* (2007)
MEK1 (MAP2K1)	Putative regulation of MAPKK activity	• Activation or inhibition of MAPK pathways, depending on TRIB3 dose	Kiss-Toth *et al.* (2004)
MKK7 (MAP2K7)	Putative regulation of MAPKK activity	• Activation or inhibition of MAPK pathways, depending on TRIB3 dose	Kiss-Toth *et al.* (2004)
MLK3 (MAP3K11)	Stabilization of TRIB3 and MLK3 proteins	• Enhanced Akt inhibition by TRIB3 • Promotion of apoptosis	Humphrey *et al.* (2010); Humphrey *et al.* (2014)
mTOR	Inhibition of mTORC2 activity	• Reduced Akt S473 phosphorylation • Resistance to diabetic kidney disease	Borsting *et al.* (2014)

2.4.1.4. TRIB3 can directly affect NF-κB activity in cells subjected to stress

TRIB3 can also contribute to cell death by regulating NF-κB, a central transcription factor for the promotion of inflammatory responses, cell survival and cell proliferation (Fang *et al.*, 2014; Rzymski *et al.*, 2008; Wu *et al.*, 2003).

TRIB3 physically interacts with the NF-κB member RelA, a transcription activator, and can inhibit NF-κB activity (Duggan *et al.*, 2010; Rzymski *et al.*, 2008; Smith *et al.*, 2011; Wu *et al.*, 2003). TRIB3 reduces RelA phosphorylation by protein kinase A, a mechanism for the activation of NF-κB, but does not repress the nuclear translocation of RelA or RelA binding to DNA (Wu *et al.*, 2003). As NF-κB can contribute to the upregulation of *TRIB3* expression in response to cellular stress and cytokines, TRIB3 functions as a negative feedback regulator of NF-κB activity (Rzymski *et al.*, 2008; Wu *et al.*, 2003). Additionally, TRIB3 can inhibit NF-κB as a downstream effector of the ATF4–CHOP pathway (Rzymski *et al.*, 2008; Smith *et al.*, 2011).

TRIB3 has been found to accelerate cell death by repressing NF-κB (Rzymski *et al.*, 2008; Wu *et al.*, 2003); however, recently there have also been reports that TRIB3 can increase NF-κB activity (Fang *et al.*, 2014; Yu *et al.*, 2015). Nevertheless, the effect of TRIB3 on cell survival was found to be negative in such a case as well, since, unusually, NF-κB activity aggravates cell death induced by ER stress in pancreatic β-cells (Fang *et al.*, 2014).

2.4.1.5. TRIB3: a caspase-3 substrate and regulator

Caspases are a family of aspartate-directed cysteine proteases that are responsible for carrying out apoptotic cell death, and they function in a hierarchical manner, in which 'apical' (initiator) caspases cleave the inactive pro-forms of 'executioner' (effector) caspases, thereby activating the latter, which then proceed to cleave other cellular proteins and irreversibly commit the cell to apoptosis (Inoue *et al.*, 2009).

Human TRIB3 is a substrate for at least seven caspases, including multiple initiator and effector caspases, and is implicated in the regulation of cell death by modulating caspase activity (Shimizu *et al.*, 2012). Caspases 3, 6, 7, 8, 9 and 10 proteolytically cleave TRIB3 at a site located twenty amino acid residues from the C-terminus, while caspase-2 cleaves more close to the C-terminus (Shimizu *et al.*, 2012).

In cells subjected to apoptosis-inducing treatment, the cleavage of TRIB3 expedites apoptosis and augments the activation of executioner caspases 3 and/or 7 (Shimizu *et al.*, 2012). However, under certain stress conditions, for instance tunicamycin-induced ER stress in HeLa cells, TRIB3 was found to be uncleaved, even though caspase-3/7 activation was detected and the mode of cell death was predominantly apoptotic (Shimizu *et al.*, 2012). In this case, TRIB3 (herein uncleaved) suppressed caspase-3/7 activation and asserted an anti-apoptotic effect on cell viability, possibly since TRIB3 promoted the nuclear translocation of

procaspase-3, which appeared to preclude caspase-3 activation (Shimizu *et al.*, 2012). Thus, TRIB3 can serve either a pro- or anti-apoptotic role by regulating caspase activation, and the additional factors that determine the direction of the effect of TRIB3 in this context are currently unknown.

2.4.2. TRIB3 is involved in protein degradation

The prototypical member of the Tribbles protein family, *Drosophila* Tribbles, was originally found to exert its effects by targeting specific proteins for ubiquitination, leading to their destruction by the proteasome (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Rorth *et al.*, 2000; Seher and Leptin, 2000), and this mode of function is also used by mammalian TRIB3 in a variety of different contexts. Accompanying this, TRIB3 is itself subject to ubiquitination-mediated degradation. In addition to the ubiquitin–proteasome system, TRIB3 is also associated with regulating autophagy, the other main route of intracellular protein degradation.

2.4.2.1. TRIB3 is linked to TGF-β and BMP signaling by E3 ubiquitin ligases SMURF1 and SMURF2

The proteins SMURF1 ('SMAD-specific E3 ubiquitin protein ligase 1') and SMURF2 are E3 ubiquitin ligases that participate in receptor-mediated signaling by regulating the degradation of SMAD proteins, the intracellular signal transducers for transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP) signaling, though many other targets have also been identified for SMURF1/2 (David *et al.*, 2013; Zhu *et al.*, 1999). TRIB3 interacts with both SMURF1 and SMURF2, and, in both cases, TRIB3 induces the degradation of the SMURF protein by the ubiquitin–proteasome pathway (Chan *et al.*, 2007; Hua *et al.*, 2011).

Since SMURF2 promotes the proteasomal degradation of SMAD2 and SMAD3, two downstream transducers of TGF-β signaling, TRIB3 is able to augment TGF-β–SMAD signaling by decreasing the abundance of SMURF2 (Hua *et al.*, 2011). In addition to reducing the degradation rate of SMAD2/3, TRIB3 further enhances SMAD3 transcriptional activity by interacting with SMAD3 and promoting its nuclear localization (Hua *et al.*, 2011). Moreover, a positive feedback loop may exist between TRIB3 and TGF-β–SMAD3 signaling, since *TRIB3* expression is upregulated in response to stimulation with TGF-β or the overexpression of SMAD3 (Hua *et al.*, 2011).

SMURF1 degradation is triggered by TRIB3 in response to simulation with BMP (Chan *et al.*, 2007). TRIB3 binds to the cytoplasmic region of the BMP type II receptor (BMPR2) in unstimulated cells, but dissociates upon BMP4 binding and proceeds to induce proteasomal degradation of SMURF1 (Chan *et al.*, 2007). By promoting the destruction of SMURF1, TRIB3 has been shown to decrease SMURF1-mediated degradation of SMAD1, SMAD7 and RhoA, thereby positively modulating BMP, TGF-β and Rho kinase signaling, respectively (Chan *et al.*, 2007).

2.4.2.2. TRIB3 interacts with several other E3 ubiquitin ligases as well

As mentioned earlier, one of the originally discovered functions of *Drosophila* Tribbles is triggering the ubiquitination-mediated degradation of the cell cycle-regulating phosphatase String, a homolog of mammalian CDC25 proteins (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000). Possibly related to this in an evolutionary sense, TRIB3 has been reported to bind to CDC25A and positively or negatively regulate its stability, although the significance of this TRIB3 interaction for the regulation of cell cycle progression by CDC25A is unknown (Sakai *et al.*, 2010). The results of Sakai *et al.* (2010) demonstrate that TRIB3 increases CDC25A ubiquitination and degradation under normal growth conditions but stabilizes CDC25A in response to DNA damage. Two ubiquitin ligase complexes, $SCF^{\beta\text{-}TrCP}$ and APC/C^{CDH1}, previously known to induce CDC25A breakdown, are implicated in mediating the destabilizing effect of TRIB3 on CDC25A (Sakai *et al.*, 2010).

Interestingly, the APC/C^{CDH1} ubiquitin ligase complex is also involved in the ubiquitination and turnover of TRIB3 *via* an interaction between TRIB3 and CDH1, the co-activator protein of APC/C, indicating a possible regulatory network involving TRIB3, CDC25A and APC/C^{CDH1} (Ohoka *et al.*, 2010; Sakai *et al.*, 2010). Another E3 ubiquitin ligase, SIAH1, has also been found to interact with and ubiquitinate TRIB3, thereby accelerating the turnover of TRIB3 protein (Zhou *et al.*, 2008b).

All three Tribbles homologs present in mammals are able to physically interact with the E3 ubiquitin ligase COP1, although the proteins targeted for degradation *via* COP1 binding differ somewhat between the different mammalian Tribbles proteins (Dedhia *et al.*, 2010; Keeshan *et al.*, 2010; Qi *et al.*, 2006; Yokoyama *et al.*, 2010; Yoshida *et al.*, 2013). Whereas TRIB1 and TRIB2 are efficient at triggering the degradation of C/EBPα, a transcription factor that promotes myeloid differentiation, such an ability is absent in the case of TRIB3 (Dedhia *et al.*, 2010). Due to this functional difference, unrestrained expression of TRIB1 or TRIB2 induces leukemia, while the misexpression of TRIB3 does not (Dedhia *et al.*, 2010; Keeshan *et al.*, 2006; Yokoyama *et al.*, 2010; Yoshida *et al.*, 2013). TRIB1, 2 and 3 all appear to be able to direct COP1 to target acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis, for proteasomal destruction (Dedhia *et al.*, 2010). However, this function has most thoroughly been validated in the case of overexpressed TRIB3 (Qi *et al.*, 2006).

Recent results reveal that TRIB3 binds to a further E3 ubiquitin ligase, Parkin, which is a protein that protects against Parkinson's disease by facilitating the clearance of pathogenic substrates (Aime *et al.*, 2015). TRIB3 expression leads to decreased Parkin protein level, without affecting the mRNA level, and enhances cell death in cellular models of Parkinson's disease (Aime *et al.*, 2015).

Table 5. Ubiquitin ligases reported to physically interact with TRIB3. Only interactions with known functional significance are included in this table.

Protein	Result of interaction	Biological significance	Reference
APC/C^{CDH1} (FZR1)	Ubiquitination of TRIB3 by APC/C	• Decreased half-life of TRIB3 protein due to proteasomal degradation	Ohoka *et al.* (2010)
COP1 (RFWD2)	Ubiquitination of ACC1 by COP1, proteasomal degradation of ACC1	• Decreased fatty acid synthesis • Resistance to diet-induced obesity	Qi *et al.* (2006)
Parkin (PARK2)	Decreased Parkin protein level	• Promotion of cell death in cellular models of Parkinson's disease	Aime *et al.* (2015)
SIAH1	Ubiquitination of TRIB3 by SIAH1	• Decreased half-life of TRIB3 protein due to proteasomal degradation	Zhou *et al.* (2008b)
SMURF1	Ubiquitination and proteasomal degradation of SMURF1	• Decreased SMURF1-mediated degradation of SMAD1, SMAD7 and RhoA • Activation of BMP and TGF-β signaling	Chan *et al.* (2007)
SMURF2	Ubiquitination and proteasomal degradation of SMURF2	• Decreased SMAD3 ubiquitination by SMURF2 • Activation of TGF-β–SMAD signaling • Promotion of tumor cell migration and invasion	Hua *et al.* (2011)

2.4.2.3. TRIB3 in autophagy: a dichotomy

In addition to interacting with ubiquitin ligases, TRIB3 is implicated in regulating protein degradation on a more general level by negatively affecting the clearance of autophagic and proteasomal substrates (Hua *et al.*, 2015b). Sequestosome 1 (SQSTM1), frequently known as p62, is a selective autophagy receptor that binds to ubiquitinated proteins and targets them to autophagosomes for subsequent degradation (Rogov *et al.*, 2014). TRIB3 binds to SQSTM1 and blocks its function, resulting in the attenuation of autophagic flux and the accumulation of SQSTM1 (Hua *et al.*, 2015b). The accumulation of SQSTM1 underlies the inhibition of the ubiquitin–proteasome system by TRIB3, possibly due to competition between proteasomal delivery receptors and SQSTM1 for the binding of ubiquitinated substrates, which leads to

Table 6. TRIB3-interacting proteins of various functional categories, except transcription factors (presented separately in Table 3), protein kinases (Table 4) and ubiquitin ligases (Table 5). Only interactions with known biological significance are included in this table.

Protein	Result of interaction	Biological significance	Reference(s)
ACC1/2 (ACACA, ACACB)	Ubiquitination of ACC by COP1, proteasomal degradation of ACC	• Decreased fatty acid synthesis • Resistance to diet-induced obesity	Qi *et al.* (2006)
APOBEC3A	Possible reduction of APOBEC3A abundance	• Possible decrease in nuclear DNA editing and increase in genome integrity	Aynaud *et al.* (2012); Land *et al.* (2013)
Caspase-3	Cleavage of TRIB3 at position D338	• Suppression of apoptosis by uncleaved TRIB3 *via* prevention of caspase-3 activation	Shimizu *et al.* (2012)
CDC25A	Regulation of ubiquitination-mediated CDC25A degradation	• Differential CDC25A stability in normal and genotoxic conditions	Sakai *et al.* (2010)
CtIP (RBBP8)	Unknown/binding of TRIB3 and CtIP	• UV-sensitive dot-like nuclear co-localization of TRIB3 and CtIP	Xu *et al.* (2007)
IRS1	Inhibition of IRS1 phosphorylation at Y612	• Inhibition of proximal insulin signaling	Koh *et al.* (2013)
PCAF (KAT2B)	Unknown (possibly the acetylation of TRIB3)	• TRIB3 hypo-acetylation may contribute to its Akt-inhibiting activity	Yao and Nyomba (2008)
RICTOR	Inhibition of mTORC2 activity	• Reduced Akt S473 phosphorylation • Resistance to diabetic kidney disease	Borsting *et al.* (2014)
SMYD1	Methylation of TRIB3	• Enhanced SMYD1-mediated repression of transcription by methylated TRIB3 • Potential positive feedback regulation of SMYD1 activity	Rasmussen *et al.* (2015)
SQSTM1 (p62)	Inhibition of SQSTM1 binding to LC3; accumulation of SQSTM1	• Attenuation of autophagic and proteasomal degradation • Promotion of tumor cell proliferation and invasion • Promotion of tumor growth, metastasis and recurrence	Hua *et al.* (2015b)

ubiquitinated proteins being denied access to the proteasome (Hua *et al.*, 2015a; Hua *et al.*, 2015b). Consequently, the two main pathways for the degradation of intracellular proteins, the autophagy–lysosome route and the ubiquitin–proteasome system, can both be inhibited by TRIB3, and, through this, TRIB3 has been linked to accelerated tumor progression in mouse models (Hua *et al.*, 2015b).

On the other hand, TRIB3 has also been reported to stimulate autophagy by inhibiting Akt, thereby leading to mTORC1 inactivation, a common molecular event for triggering autophagy (Salazar *et al.*, 2009b; Salazar *et al.*, 2013; Vara *et al.*, 2011). This mode of TRIB3 action is necessary for cannabinoid-induced autophagy in glioma and hepatoma cells, and represents a potential mechanism of anti-cancer therapy (Salazar *et al.*, 2009b; Vara *et al.*, 2011). Thus, in different situations, TRIB3 may apparently have opposing effects on autophagy.

2.4.3. TRIB3 in development and differentiation

2.4.3.1. *Trib3*-deficient mice appear normal under standard conditions

Knockout mice provide valuable information about the functions of a gene at endogenous levels of expression. As such, mice with a targeted disruption of the *Trib3* gene have been generated and studied by several groups (Table 7). Additionally, several transgenic mouse lines with tissue-specific overexpression of *Trib3* have also been created and characterized (Table 8).

Homozygous *Trib3* knockout ($Trib3^{-/-}$) mice are viable and physically grossly normal, and the crossing of *Trib3* heterozygotic ($Trib3^{+/-}$) individuals leads to all genotypes being born in the expected Mendelian ratio (Okamoto *et al.*, 2007). These results resemble what has been observed in mice with a deletion of *Trib1* or *Trib2* (Takasato *et al.*, 2008; Yamamoto *et al.*, 2007). The International Mouse Phenotyping Consortium has recently released phenotyping data from the analysis of adult *Trib3* knockout mice, including the characterization of morphology, basic physiology, clinical chemistry, hematology and basic behavioral responses, and currently no significant phenotype associations have been found, although the statistical power may be limited in some cases (data available at: www.mousephenotype.org, accessed 2015-10-29, database release 3.4; Koscielny *et al.*, 2014).

It has been postulated that in the single-knockout mouse lines, other Tribbles homologs might functionally compensate for the lack of one Tribbles family member (Takasato *et al.*, 2008). However, the typical expression patterns of the different Tribbles family members overlap only partially (as discussed in section 2.3.1), and, in $Trib3^{-/-}$ mice, unaltered expression of *Trib1* and *Trib2* was observed in the liver, white adipose tissue, brown adipose tissue and skeletal muscle (Okamoto *et al.*, 2007). Moreover, functional redundancy would seem more probable between *Trib1* and *Trib2* than between either of them and *Trib3*, since the amino acid sequences of TRIB1 and TRIB2 are considerably more similar to each other than to TRIB3 (as described in section 2.2.3). Nevertheless, the creation of double- or triple-knockout mouse lines could yield new insights into the functions of the Tribbles homologs in mammals.

Table 7. Characteristics of *Trib3*-deficient mice.

Phenotype of *Trib3* knockout mice	Reference
• Viable, physically grossly normal, born in the expected Mendelian ratio • Unmodified insulin and glucose tolerance • Normal hepatic insulin signaling, serum biochemistry, body composition and metabolic rate	Okamoto *et al.* (2007)
• Normal splenic macrophages and lymphocytes	Satoh *et al.* (2013)
• On a standard rodent chow diet: unaltered body weight, blood glucose level and glucose tolerance • On a high-fat diet: improved glucose tolerance, less body weight gain, lower fasting levels of serum glucose and insulin, decreased liver weight and triglyceride content, unaltered fat pad weight	Koh *et al.* (2013)
• Unaltered developmental apoptosis of superior cervical ganglion neurons	Zareen *et al.* (2013)
• Normal baseline kidney function and structure • With streptozotocin-induced diabetes: increased severity of diabetic kidney disease	Borsting *et al.* (2014)
• No effect on pancreatic β-cell mass and size • Normal glucose tolerance, growth and body weight	Humphrey *et al.* (2014)
• Low, unaltered baseline incidence of tissue lesions in several organs at 8 months of age • In a chemical carcinogenesis model of skin cancer: accelerated malignant progression • In a genetic model of cancer predisposition: increased incidence of premalignant and malignant lesions in several organs	Salazar *et al.* (2015)
• No evident alterations in the anatomy or cellular content of the cortical and subcortical regions of the brain	Aime *et al.* (2015)
• Enhanced insulin signaling in brown adipose tissue	Jeong *et al.* (2016)
• International Mouse Phenotyping Consortium: no statistically significant phenotype associations found for adult mice in analyses that span morphology, basic physiology, clinical chemistry, hematology and basic behavioral experiments (data available at: www.mousephenotype.org, accessed 2015-10-29)	International Mouse Phenotyping Consortium (database release 3.4; Koscielny *et al.*, 2014)

Since previous work by Du *et al.* (2003) had implicated TRIB3 in insulin signaling in the liver, an organ with a principal role in glucose homeostasis, Okamoto *et al.* (2007) carried out a thorough characterization of the glucose and energy metabolism of adult *Trib3*$^{-/-}$ mice maintained under standard conditions

(*e.g.*, feeding with a standard rodent chow diet *ad libitum*). The results revealed that *Trib3*-deficient mice have normal serum metabolic parameters and body composition, as well as unaltered hepatic insulin signaling and glucose metabolism (Okamoto *et al.*, 2007). Additionally, no discernible effect in insulin or glucose tolerance was found compared to wild type controls, and whole-body metabolic rate and energy expenditure were also not affected by the deletion of *Trib3* (Okamoto et al., 2007). Adequate production of insulin by pancreatic β-cells is also critical for the control of glucose homeostasis, and an analysis of *Trib3* knockout mice has revealed that they have no apparent differences in pancreatic β-cell mass and size (Humphrey *et al.*, 2014). Thus, the global loss of *Trib3* does not appear to affect the maintenance of glucose homeostasis in mice maintained under standard conditions.

Further studies performed with *Trib3* knockout mice have revealed that *Trib3*$^{-/-}$ individuals also have normal baseline kidney function and structure (Borsting *et al.*, 2014) and normal macrophage and lymphocyte populations in the spleen (Satoh *et al.*, 2013).

Consistent with the results obtained from *Trib3* knockouts, the analysis of transgenic mice that conditionally overexpress human *TRIB3* in a Cre recombinase-dependent manner revealed that ten days of enforced *TRIB3* expression in the liver, spleen and kidney causes no apparent histological differences in kidney and spleen (Sakai *et al.*, 2014). However, in the mouse liver, the Cre-mediated overexpression of human *TRIB3* gave rise to an increase in hepatocyte nuclear size as well as slight inflammation in the perivascular regions, compared to Cre-treated wild type mice (Sakai *et al.*, 2014).

2.4.3.2. Transgenic models implicate TRIB3 in myocyte physiology

Transgenic mice that overexpress *Trib3* in a skeletal muscle-specific manner display a dramatically increased endurance exercise capacity and a pronounced increase in the proportion of oxidative muscle fibers (An *et al.*, 2014). No difference in their overall body weight is evident, though the weights of individual muscles tend to be elevated compared to wild type controls (An *et al.*, 2014). Metabolically, *Trib3* overexpression in skeletal muscle has no effect on muscle or whole-body glucose homeostasis; however, *in vitro* analysis of the transgenic muscle revealed decreased basal but unaltered contraction-stimulated fatty acid oxidation (An *et al.*, 2014).

Transgenic mice that overexpress *Trib3* specifically in cardiac muscle have also been generated, and these mice develop hearts with normal size and contractile function (Avery *et al.*, 2010). However, metabolically, *Trib3* overexpression in the heart leads to a reduced glucose oxidation rate without affecting the rate of fatty acid oxidation (Avery *et al.*, 2010). Comparably to *Trib3*-overexpressing skeletal muscle, *Trib3*-overexpressing hearts display elevated expression of slow-twitch myosin, which is associated with oxidative skeletal muscle fibers (An *et al.*, 2014; Avery *et al.*, 2010). Recently, it has been

demonstrated that TRIB3 interacts with and functions as a transcription co-repressor for SMYD1, a factor required for embryonic heart development (Rasmussen *et al.*, 2015), though whether this is associated with the phenotype of the mice that overexpress *Trib3* in cardiac muscle remains unknown. Overall, these results indicate that TRIB3 is able to influence muscle composition in skeletal as well as cardiac muscle.

Table 8. Characteristics of transgenic mice that overexpress TRIB3.

Genetic modification	Phenotype of mutant mice	Reference
Adipose tissue-specific overexpression of *Trib3*	• Less white adipose tissue, slower weight gain • Increased dietary intake • Blood glucose level decreased, fatty acid level increased • Protection from diet-induced obesity	Qi *et al.* (2006)
Cardiac muscle-specific overexpression of *Trib3*	• Normal baseline heart size and contractile function • Increased heart damage after myocardial infarction	Avery *et al.* (2010)
Pancreatic β-cell-specific overexpression of *Trib3*	• At 4 months of age: unaltered body weight and insulin sensitivity • At older ages: decreased glucose tolerance, insulin secretion, pancreatic β-cell mass and proliferation	Liew *et al.* (2010)
Skeletal muscle-specific overexpression of *Trib3*	• Greatly increased endurance exercise capacity • Increased proportion of oxidative muscle fibers • Normal muscle and whole-body glucose homeostasis	An *et al.* (2014)
Conditional overexpression of human *TRIB3*	• Ten days after adenoviral delivery of Cre recombinase: increased hepatocyte nuclear size, slight perivascular inflammation in the liver, unaltered kidney and spleen histology (compared to Cre-treated wild type)	Sakai *et al.* (2014)

In vitro models also implicate TRIB3 in the regulation of vascular smooth muscle cell phenotype due to its ability to trigger BMP-induced degradation of SMURF1 (Chan *et al.*, 2007). In response to BMP stimulation of vascular smooth muscle cells, microRNA miR-96 is repressed, leading to the derepression of *Trib3* and thereby to the promotion of a contractile phenotype (Kim *et al.*, 2014b). In

contrast, platelet-derived growth factor (PDGF)-BB stimulation of vascular smooth muscle cells downregulates *Trib3 via* the induction of miR-24, leading to the promotion of a proliferative phenotype (Chan *et al.*, 2010). Thus, *Trib3* is linked to myocyte function in skeletal, smooth and cardiac types of muscle.

2.4.3.3. A potential role for TRIB3 in adipose tissue

On a standard chow diet, mice with an adipose tissue-specific overexpression of *Trib3* gain weight more slowly than their wild type littermates and display a reduced amount of white adipose tissue relative to body weight, even though they actually consume more calories (Qi *et al.*, 2006). Accompanying this, adipocyte size is decreased in the *Trib3*-overexpressing adipose tissue, and the transgenic mice additionally exhibit lower glucose but higher fatty acid concentrations in blood on a standard chow diet (Qi *et al.*, 2006). *Trib3*-overexpressing adipocytes were found to display an increased fatty acid oxidation rate and reduced activity of ACC, the rate-limiting enzyme in fatty acid synthesis (Qi *et al.*, 2006). Mechanistically, TRIB3-induced ubiquitination of ACC by the ubiquitin ligase COP1 (discussed in section 2.4.2.2) is thought to underlie the phenotype of the mice with adipose tissue-specific overexpression of *Trib3* (Qi *et al.*, 2006).

In addition to the inhibitory effect of *Trib3* overexpression on fat accumulation into adipocytes, TRIB3 is also implicated in the suppression of multiple stages of adipocyte differentiation, based on *in vitro* models of adipogenesis (Bezy *et al.*, 2007; Jeong *et al.*, 2016; Naiki *et al.*, 2007; Takahashi *et al.*, 2008). In the early stages of adipocyte differentiation, *Trib3* expression is temporarily suppressed, which is necessary to relieve the inhibitory effect of TRIB3 on C/EBPβ and PPARγ, two transcriptional regulators that drive consecutive steps of the adipogenic differentiation cascade (Bezy *et al.*, 2007; Takahashi *et al.*, 2008). As with C/EBPβ (discussed in section 2.4.1.2), TRIB3 inhibits PPARγ by binding to it and repressing its transcriptional activity (Takahashi *et al.*, 2008). The downregulation of *Trib3* in early adipogenesis also facilitates the activation of Akt kinase, a key event in adipocyte differentiation (Naiki *et al.*, 2007). Since the adipose tissue-specific *Trib3* overexpression mouse line generated by Qi *et al.* (2006) utilizes a promoter construct that is only strongly activated starting from late adipogenesis, the phenotype of these mice is probably not informative with regard to the observed functions of *Trib3* in adipocyte differentiation (Takahashi *et al.*, 2008).

In light of these results, it is notable that standard diet-fed *Trib3* knockout mice display unaltered body weight and body fat percentage, and their serum levels of triglycerides and free fatty acids are indistinguishable from wild type controls (Okamoto *et al.*, 2007). Similarly, no difference in the protein level of ACC in adipose tissue was observed in standard chow diet-fed mice lacking *Trib3* (Okamoto *et al.*, 2007). Thus, *Trib3* does not appear to have a non-redundant role in mouse adipogenesis or fat metabolism in standard laboratory animal husbandry conditions.

2.4.3.4. Links between TRIB3 and the hematopoietic system

There are multiple reports assigning different roles to TRIB3 in hematopoietic cells, and *TRIB3* demonstrates interesting expression dynamics in hematopoietic processes; however, a comprehensive picture has yet to emerge. For example, in *ex vivo* models of erythropoiesis, *Trib3* is upregulated in erythroblasts in response to the cytokine erythropoietin and promotes erythroid progenitor cell survival (da Cunha *et al.*, 2010; Dev *et al.*, 2010; Sathyanarayana *et al.*, 2008; Singh *et al.*, 2012). In contrast, in an *ex vivo* model of human megakaryopoiesis, *TRIB3* expression is repressed during the course of megakaryocyte differentiation (Ahluwalia *et al.*, 2015).

Regarding the very early stages of hematopoietic differentiation, the downregulation of *Trib3* appears to be a requirement for the *in vitro* differentiation of hematopoietic progenitors from mouse embryonic stem cells (Roy *et al.*, 2015). Going a stage forward from hematopoietic progenitors, to the myeloid commitment of mouse hematopoietic stem cells, involves a marked increase in *Trib3* expression (Klimmeck *et al.*, 2014).

As mentioned in section 2.4.2.2, *TRIB3* is not a leukemia oncogene, as opposed to *TRIB1* and *TRIB2*, since TRIB3 does not trigger ubiquitination and degradation of C/EBPα, a transcription factor involved in myeloid differentiation (Dedhia *et al.*, 2010; Keeshan *et al.*, 2006; Yokoyama *et al.*, 2010; Yoshida *et al.*, 2013). Transplantation of bone marrow overexpressing *Trib1* or *Trib2* into lethally irradiated mice leads to the development of acute myeloid leukemia, while mice transplanted with *Trib3*-overexpressing bone marrow do not display leukemogenesis and present normal spleen weight, white blood cell count, bone marrow blast proportion and overall survival (Dedhia *et al.*, 2010).

2.4.3.5. TRIB3 and the brain

Little is known about the physiological importance of *TRIB3* in the brain. Recent studies have reported that *Trib3*-deficient mice display no apparent alterations in the developmental apoptosis of neurons and present grossly normal anatomy and cellular content in the cortical and subcortical regions of the brain (Aime *et al.*, 2015; Zareen *et al.*, 2013). A human single-nucleotide polymorphism (dbSNP ID: rs6051520) located between *TRIB3* and its neighboring gene, *NRSN2*, has been associated with information processing speed in a genome-wide association study (Luciano *et al.*, 2011). The polymorphism is quite common, with a minor allele frequency slightly above 0.2 in the Caucasian cohorts studied by Luciano *et al.* (2011), and is located approximately 9 kbp from the 5′-end of *TRIB3* and approximately 16 kbp from the 3′-end of *NRSN2*. This polymorphism is also noteworthy because it is currently the only genome-wide association linked to *TRIB3*, according to the NHGRI-EBI Catalog of Published Genome-wide Association Studies (available at: www.ebi.ac.uk/gwas, accessed 2015-10-15; Welter *et al.*, 2014).

2.4.3.6. Heterozygous *TRIB3* loss-of-function appears to be tolerated in humans

Through the ongoing progress of large-scale high-throughput sequencing projects, sufficient numbers of human exomes have now been acquired to evaluate which genes are subject to strong selective pressure against protein-altering variants in the human population (Lek *et al.*, 2015). In particular, the Exome Aggregation Consortium has presented an analysis of the genetic variance in the exome sequences of nearly 61,000 unrelated adults, with individuals affected by severe pediatric diseases excluded from the sample (data available at: exac.broadinstitute.org, accessed 2015-11-17; Lek *et al.*, 2015). In this dataset, the number of loss-of-function, missense and synonymous variants observed in the *TRIB3* gene corresponds closely to the number of variants expected by random mutation, indicating that a heterozygous loss of *TRIB3* function is tolerable in humans. No individuals with a disruption of both *TRIB3* alleles (*i.e.*, knockout individuals) have been identified by the Exome Aggregation Consortium or by studies aimed at identifying human knockouts in specific populations (Narasimhan *et al.*, 2016; Sulem *et al.*, 2015); however, this is to be expected, based on the sample sizes used in the analyses and the allele frequencies of the observed *TRIB3* loss-of-function alleles.

Similarly, no *TRIB1* or *TRIB2* knockout humans have been reported by these studies (Lek *et al.*, 2015; Narasimhan *et al.*, 2016; Sulem *et al.*, 2015). Remarkably though, in contrast to *TRIB3*, there appears to be a considerable intolerance towards a heterozygous loss of *TRIB1* or *TRIB2* in humans. The Exome Aggregation Consortium data (referenced above) reveals that *TRIB2* is a possible haplo-insufficient gene, and *TRIB1* loss-of-function variants also appear to be markedly under-represented. In the case of *TRIB3*, it can be proposed that the loss of one allele can be masked by the remaining functional allele, due to the existence of several negative feedback mechanisms that regulate *TRIB3* transcription (Table 3). Additionally, since both healthy and diseased individuals (except severe pediatric disorder patients, as mentioned above) were included in the sample of exomes investigated by the Exome Aggregation Consortium, future studies that take into account disease-state may uncover associations between *TRIB3* coding variation and disease susceptibility.

2.4.4. *TRIB3*—a tumor suppressor or tumor-promoting gene?

Multiple lines of evidence link TRIB3 to cancer development, progression and metastasis. However, there are also indications that TRIB3 may act as an anti-tumor factor. In light of such apparently contradictory results, the question stated in the heading of this section arises. Which way is it then, is *TRIB3* a tumor promoter or suppressor? Intriguingly, current research suggests that a single answer may indeed be inadequate; *TRIB3* could be both.

2.4.4.1. *TRIB3* is overexpressed in several types of cancer and its expression level is linked to disease prognosis

Overexpression of *TRIB3* compared to normal tissue has been reported in many types of human tumors. TRIB3 protein levels have been found to be elevated in colorectal, liver and lung cancer (Hua *et al.*, 2015b; Miyoshi *et al.*, 2009; Zhou *et al.*, 2013a), and *TRIB3* mRNA overexpression has been reported in colorectal, breast, lung, uterine, ovarian and esophagus cancer (Bowers *et al.*, 2003; Miyoshi *et al.*, 2009; Xu *et al.*, 2007; Zhou *et al.*, 2013a).

Significantly, a high level of intra-tumor TRIB3 protein expression is associated with poor prognosis in colorectal, liver and lung cancer patients (Hua *et al.*, 2015b), and high intra-tumor *TRIB3* mRNA expression is correlated with poor prognosis in patients with colorectal, lung or breast cancer (Miyoshi *et al.*, 2009; Wennemers *et al.*, 2011a; Wennemers *et al.*, 2011b; Zhou *et al.*, 2013a). Curiously, while high *TRIB3* mRNA expression was found to be associated with poor prognosis, a high level of TRIB3 protein expression was found to denote good prognosis in the same cohort of breast cancer patients (Wennemers *et al.*, 2011b). As mentioned in section 2.2.2, TRIB3 is post-translationally regulated by variation of its protein stability, depending on the cellular context. In the case of breast cancer patients, the research shows that TRIB3 mRNA and protein levels in tumor material often do not correlate with each other (Wennemers *et al.*, 2011b; Wennemers *et al.*, 2012). On the other hand, in colorectal cancer patients, an association exists between the levels of TRIB3 mRNA and protein in tumor samples (Miyoshi *et al.*, 2009).

As with many regions of human chromosome 20, the *TRIB3* gene locus is relatively frequently increased in copy number in colorectal tumors; for instance, *TRIB3* copy number gain was detected in 10 out of 64 colorectal cancer samples (Ali Hassan *et al.*, 2014) and in 16 out of 40 colon tumor samples (Loo *et al.*, 2013). Thus, gains in *TRIB3* gene copy number could contribute to increased *TRIB3* expression in some tumors and may represent an advantageous genomic alteration for cancer cells, taking into account the association between *TRIB3* expression level and poor prognosis that has been reported in several types of cancer (referenced above).

In general, proto-oncogenes can become oncogenes due to increased expression levels or protein-altering mutations (Kumar *et al.*, 2015). In an analysis of somatic point mutations in nearly 5,000 human cancer exomes, the *TRIB3* coding sequence in cancers was not mutated significantly differently from the background expectation (data available at: www.tumorportal.org, accessed 2015-10-15; Lawrence *et al.*, 2014). However, Lawrence *et al.* (2014) do note that the power of their analysis is considerably limited by the currently available sample size; thus, while somatic alterations to the TRIB3 protein sequence are not currently suspected to be clinically relevant to cancer, this view may need to be revised following future studies.

2.4.4.2. TRIB3 as a modulator of the cellular stress response in tumor tissue

The interior regions of solid tumors often represent a harsh microenvironment in which cells suffer from a multitude of different stresses, including hypoxia, nutrient deprivation and ER stress (Fukumura *et al.*, 2010; Hirayama *et al.*, 2009; Tameire *et al.*, 2015). This may underlie the overexpression of *TRIB3* in tumors, since such stresses lead to the transcriptional induction of *TRIB3* (discussed in section 2.3). Subsequently, stress-induced TRIB3 can potentially regulate the cellular stress response and positively or negatively affect cell viability by the mechanisms described in section 2.4.1. For example, TRIB3 expression in breast cancer tissue co-localizes with hypoxic regions, and, in breast cancer cells, the knockdown of *TRIB3* expression decreases resistance to hypoxia (Wennemers *et al.*, 2011a). In another instance, glutamine deficiency occurring in *in vivo* models of neuroblastoma leads to the induction of *TRIB3*, and the increased level of TRIB3 participates in the promotion of cell death and thereby inhibits tumor growth (Qing *et al.*, 2012).

2.4.4.3. TRIB3 may suppress tumor initiation

By studying *Trib3* knockout mice using multiple models of tumorigenesis, Salazar *et al.* (2015) have demonstrated that endogenous TRIB3 suppresses tumor formation. In a tumor engraftment experiment using Ras/E1A-transformed mouse embryonic fibroblasts, the transplantation of $Trib3^{-/-}$ cells result in the accelerated onset of tumor growth, compared to $Trib3^{+/+}$ cells (Salazar *et al.*, 2015). Using an *in vivo* chemical carcinogenesis model of skin cancer development, Salazar *et al.* (2015) also show that malignant skin lesions arise with greater frequency in *Trib3*-deficient mice. Further, by crossing *Trib3* knockout mice with mice genetically predisposed to tumor development due to mono-allelic loss of PTEN ($Pten^{+/-}$ mice), it was demonstrated that the loss of *Trib3* increases the incidence of premalignant and malignant lesions on a $Pten^{+/-}$ genetic background (the whole-body loss of *Trib3* alone did not increase the baseline incidence of such lesions) (Salazar *et al.*, 2015). The anti-tumorigenic effect of *Trib3* was found to involve the suppression of Akt activation by TRIB3, and, in the absence of *Trib3*, increased Akt activity led to enhanced inactivation of FoxO3, a tumor-suppressing transcription factor (Salazar *et al.*, 2015). Thus, endogenous *Trib3* can inhibit cancer initiation by limiting Akt-driven tumorigenesis.

Pathologic conditions involving a chronic inflammatory state can eventually lead to the development of cancer in the affected part of the body; examples of such conditions include hepatitis C and B, *Helicobacter pylori* gastritis, gastric acid reflux esophagitis and inflammatory bowel disease (Kumar *et al.*, 2015). As will be described in section 2.4.5, TRIB3 may act as a suppressor of inflammation in some such conditions, thereby potentially attenuating carcinogenesis that is driven by a chronic inflammatory state.

2.4.4.4. TRIB3 may potentiate tumor progression

Countering the anti-cancer roles described in the previous section, several lines of inquiry have revealed that *Trib3* can promote tumor growth and metastasis by a number of mechanisms. Specifically, *Trib3* overexpression was found to increase tumor incidence in a chemical carcinogenesis model of lung and liver tumor formation, and, in allograft/xenograft experiments with melanoma, liver and colon cancer cells, the depletion of TRIB3 inhibited tumor growth rate as well as the formation of metastatic nodules (Hua *et al.*, 2015b). These effects were found to be linked to the suppression of autophagic flux by TRIB3 *via* an interaction between TRIB3 and SQSTM1, and treatment with a peptide engineered to disrupt the TRIB3–SQSTM1 interaction inhibited xenograft tumor growth and metastasis (Hua *et al.*, 2015b).

Endogenous *TRIB3* has also been found to increase the growth rate of human breast cancer xenograft tumors, potentially by activating Notch, a mediator of breast cancer progression, metastasis and recurrence (Izrailit *et al.*, 2013). *In vitro*, *TRIB3* knockdown has an inhibitory effect on breast cancer cell proliferation, and this effect depends on a reduction of Notch pathway activity by the *TRIB3* silencing (Izrailit *et al.*, 2013). Similarly, TRIB3 depletion in a lung cancer cell line leads to decreased cell viability and migration with a concurrent decrease in Notch expression (Zhou *et al.*, 2013a).

TRIB3 promotes Notch activity in breast cancer cell lines by activating the MAPK–ERK and TGF-β–SMAD4 pathways (Izrailit *et al.*, 2013). Interestingly, TRIB3 potentiates TGF-β signaling in breast cancer cells by stabilizing SMAD4 *via* a mechanism that is independent of SMURF1 or SMURF2, two ubiquitin ligases that TRIB3 interacts with in order to regulate several other SMAD proteins (Chan *et al.*, 2007; Hua *et al.*, 2011; Izrailit *et al.*, 2013). The regulation of MAPK–ERK activity by TRIB3 possibly takes place *via* its interactions with the MAPK cascade members MEK1 and MLK3 (Chadee and Kyriakis, 2004; Humphrey *et al.*, 2010; Kiss-Toth *et al.*, 2004).

An additional mechanism by which TRIB3 may support tumor progression is through the positive regulation of TGF-β–SMAD3 activity (Hua *et al.*, 2011). Through SMAD3, endogenous *TRIB3* enhances cell migration and invasion in *in vitro* assays, and, in hepatoma cell culture, endogenous *TRIB3* contributes to the epithelial–mesenchymal transition, a process that promotes the initiation of metastasis by enabling cancer cell invasion (Hua *et al.*, 2011).

Taken together, these results demonstrate that *Trib3* has the potential to support tumor growth and progression by mechanisms that include the attenuation of autophagy and the positive regulation of signaling pathways, such as TGF-β and Notch signaling.

2.4.4.5. The potential for cell cycle control by TRIB3

It is also possible that TRIB3 participates in cell cycle control, a major function of *Drosophila* Tribbles (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000). In different cell lines, TRIB3 has been reported to either accelerate (Hua *et al.*, 2015b; Izrailit *et al.*, 2013; Miyoshi *et al.*, 2009; Sakai *et al.*, 2013), decelerate (Salazar *et al.*, 2015; Selim *et al.*, 2007) or have no effect (Örd *et al.*, 2007; Schwarzer *et al.*, 2006) on the rate of cell proliferation.

In a case where TRIB3 was found to impede cell proliferation, the overexpression of TRIB3 induced a cell cycle delay in the G2 phase and inhibited the promoter of Cyclin B1, a factor required for transitioning through G2 (Morse *et al.*, 2009; Selim *et al.*, 2007). These effects could be mediated by the interaction between TRIB3 and CDC25A, a phosphatase that activates the G2/M cell cycle transition (Sakai *et al.*, 2010). TRIB3 also interacts with CtIP, a transcriptional co-repressor involved in cell cycle checkpoint control (Xu *et al.*, 2007).

In an instance of cell proliferation acceleration by TRIB3, the overexpression of *TRIB3* was associated with elevated Cyclin D1 and B1 expression levels and also with an increased appearance of polyploidization (Sakai *et al.*, 2013). Conversely, TRIB3 is also implicated in the maintenance of genome integrity by interacting with APOBEC3A and reducing its ability to edit nuclear DNA and induce double-strand DNA breaks (Aynaud *et al.*, 2012), though the role of TRIB3 in limiting APOBEC3A activity has been contested (Land *et al.*, 2013).

2.4.4.6. *TRIB3* may mediate cell sensitivity to chemotherapeutics

TRIB3 expression is upregulated in response to some of the chemotherapeutic compounds currently used or being investigated, as noted in section 2.3.2. Furthermore, in some cases the expression of *TRIB3* is required for the compounds to effectively kill cancer cells. TRIB3 is known to mediate the anti-cancer actions of cannabinoids in glioma and hepatocellular carcinoma cell lines by causing the inhibition of the Akt–mTORC1 axis (Salazar *et al.*, 2009b; Salazar *et al.*, 2013; Vara *et al.*, 2011). A similar TRIB3-mediated molecular mechanism is thought to underlie the cell death-inducing effect of salinomycin, a novel potent anti-cancer compound, in lung cancer cells (Li *et al.*, 2013). In both the case of cannabinoids as well as salinomycin, *TRIB3* expression is activated in response to treatment-induced ER stress (Li *et al.*, 2013; Salazar *et al.*, 2009b). Further examples of TRIB3-dependent anti-cancer therapeutics are the perifosine–sorafenib combination of kinase inhibitors in lymphoma cell lines (Locatelli *et al.*, 2013), the PPARα/γ activator ABTL0812 in lung and pancreatic cancer cell lines (Erazo *et al.*, 2015), and the NF-κB inhibitor DHMEQ in hepatoma cell lines (Lampiasi *et al.*, 2009; Lampiasi *et al.*, 2012; Lampiasi *et al.*, 2014). Notably, DHMEQ also upregulates *TRIB3* expression by inducing ER stress (Lampiasi *et al.*, 2009). TRIB3 has a dichotomous effect on

cell sensitivity to the combination of celecoxib and MG132 (inhibitors of cyclooxygenase-2 and the proteasome, respectively), since the presence of TRIB3 either increases or decreases combination-induced cell death, depending on the hepatoma cell line investigated (Cusimano *et al.*, 2010).

2.4.5. Roles for TRIB3 in inflammatory diseases

2.4.5.1. TRIB3 as an inflammation modulator through NF-κB

TRIB3 is engaged in limiting inflammatory processes by attenuating NF-κB activity (Duggan *et al.*, 2010; Olah *et al.*, 2014; Smith *et al.*, 2011), though examples of positive modulation of the NF-κB pathway by TRIB3 have also been reported (Fang *et al.*, 2014; Yu *et al.*, 2015).

Helicobacter pylori infection can lead to gastric diseases, including stomach cancer, by a process thought to involve a chronic upregulation of inflammatory mediators (Kumar *et al.*, 2015). *TRIB3* is downregulated in gastric biopsies with *H. pylori* infection, and endogenous *TRIB3* inhibits NF-κB transcriptional activity and chemokine transcription activation in response to *H. pylori* lipopolysaccharide, indicating that *TRIB3* repression by *H. pylori* could contribute to *H. pylori*-associated pathogenesis (Smith *et al.*, 2011).

Chronic reflux of gastric contents into the esophagus generates a persistent inflammatory state that may lead to the development of Barrett's esophagus, which can eventually progress to esophageal cancer (Kumar *et al.*, 2015). *TRIB3* is downregulated in Barrett's esophagus tissue samples, compared to normal esophageal tissue, and endogenous *TRIB3* inhibits NF-κB transcriptional activity as well as the expression of pro-inflammatory cytokines in esophageal cells, indicating that the decline of *TRIB3* expression in Barrett's esophagus may contribute to carcinogenesis (Duggan *et al.*, 2010).

The inhibition of NF-κB activity by TRIB3 is also thought to underlie the anti-inflammatory actions of cannabidiol, a non-psychotropic phytocannabinoid which has been studied as a potential therapeutic agent against acne vulgaris (Olah *et al.*, 2014).

Curiously, in osteoblastic cells treated with lipopolysaccharides extracted from *Porphyromonas endodontalis*, a bacterial pathogen associated with tooth pulp infections and inflammatory bone resorption, TRIB3 appears to potentiate the pro-inflammatory action of NF-κB (Yu *et al.*, 2015), indicating that the relationship between TRIB3 and NF-κB activity is complex.

2.4.5.2. Further links between TRIB3 and inflammation

TRIB3 may also participate in inflammatory signaling by differentially modulating the activation of the different MAPK classes (ERK, JNK and p38), potentially through the physical interactions between TRIB3 and the MAPKKs MEK1 and MKK7 or between TRIB3 and the MAPKKK MLK3

(Humphrey *et al.*, 2014; Kiss-Toth *et al.*, 2004). Activator protein 1 (AP-1), a sequence-specific transcription factor of heterogeneous composition (homo- and heterodimers of bZIP proteins from the Jun, Fos, Maf and ATF families), is activated in response to various extracellular stimuli through MAPK signaling cascades (Shaulian and Karin, 2002; Uluckan *et al.*, 2015). Potentially by interacting with MAPK pathway components, TRIB3 is able to inhibit AP-1 transcriptional activity in response to cytokine stimulation (Kiss-Toth *et al.*, 2004). Additionally, TRIB3 is thought to reduce pro-inflammatory cytokine gene expression in kidney cells and alleviate diabetic kidney disease by inhibiting the mTORC2–Akt pathway (Borsting *et al.*, 2014).

Systemic sclerosis is a disorder characterized by fibrosis (excessive deposition of connective tissue) in the skin and other organs of the body, and is thought to be the result of chronic inflammation originating from autoimmunity (Kumar *et al.*, 2015). Persistently activated TGF-β–SMAD signaling in fibroblasts drives the development of fibrosis, and *TRIB3*, which is overexpressed in the skin of systemic sclerosis patients, potentiates TGF-β-mediated activation of fibroblasts (Tomcik *et al.*, 2016). Further, in mouse models of systemic sclerosis, skin fibrosis can be markedly alleviated by *in vivo* knockdown of *Trib3* (Tomcik *et al.*, 2016).

In an association study based on a gene-centric microarray, a single-nucleotide polymorphism (dbSNP ID: rs6139007) located less than 1 kbp upstream of major *TRIB3* transcription initiation sites was associated with a modest effect on the concentration of circulating interleukin-6 (IL-6), a major pro-inflammatory cytokine (Shah *et al.*, 2013). Interestingly, in a genome-wide linkage analysis for the discovery of autism spectrum disorder risk loci, the chromosomal region containing *TRIB3* (20p13) revealed the strongest linkage signal, and the linkage peak is located at rs6139007 (Werling *et al.*, 2014), the same *TRIB3* upstream region variation identified in the IL-6 concentration study mentioned previously.

2.4.6. TRIB3 in diabetes and its complications

2.4.6.1. TRIB3 may contribute to insulin resistance as well as to the loss of pancreatic β-cells

Type 2 diabetes, the most prevalent type of diabetes mellitus, is characterized by insulin resistance—a reduced response to insulin in insulin-sensitive tissues, including skeletal muscle, liver and adipose tissue (Kumar *et al.*, 2015). In the aforementioned tissues, elevated *TRIB3* mRNA expression has been found in humans and rodents with insulin resistance (Bi *et al.*, 2008; Du *et al.*, 2003; Liu *et al.*, 2010; Oberkofler *et al.*, 2010). As mentioned previously (2.4.1.3), TRIB3 has been reported to inhibit Akt kinase, a critical factor for insulin signaling, and thereby contribute to insulin resistance in the liver (Du *et al.*, 2003). In the rat liver, TRIB3 is

acetylated and physically interacts with the histone acetyltransferase PCAF, and hypo-acetylation of TRIB3 has been proposed to potentiate the inhibition of Akt by TRIB3 (Yao and Nyomba, 2008).

While *Trib3*-deficient mice display no detectable aberrations in insulin signaling when maintained under standard laboratory animal husbandry conditions (as discussed in section 2.4.3.1), *Trib3* knockout mice maintained on a high-fat diet exhibit reduced skeletal muscle insulin resistance (Koh *et al.*, 2013). Similarly, *in vivo* silencing of *Trib3* expression in mouse or rat models of type 2 diabetes results in improved insulin sensitivity (Ti *et al.*, 2011; Wang *et al.*, 2012; Weismann *et al.*, 2011). Interestingly, mice engineered to overexpress *Trib3* in skeletal muscle show normal skeletal muscle insulin signaling and glucose uptake (An *et al.*, 2014).

In the insulin signal transduction cascade, the binding of insulin to the insulin receptor is followed by the activation (phosphorylation) of insulin receptor substrate (IRS) proteins, which proceed to activate further signaling pathways, such as the phosphoinositide 3-kinase (PI3K) pathway, in which Akt is considered to be the principal effector (Kumar *et al.*, 2015). Besides interacting with and inhibiting Akt, a recent report suggests that TRIB3 additionally inhibits insulin signaling upstream of Akt by interacting with and inhibiting the activation of IRS1 (Koh *et al.*, 2013).

Insulin is secreted by pancreatic β-cells, and TRIB3 has also been reported to impair insulin secretion by inhibiting insulin exocytosis (Liew *et al.*, 2010). Further, excessive *Trib3* expression can also harm pancreatic β-cell viability, as mice genetically engineered to overexpress *Trib3* in pancreatic β-cells display reduced β-cell mass and proliferation at older ages, along with decreased glucose tolerance and glucose-stimulated insulin secretion (Liew *et al.*, 2010). *Trib3* has been reported to potentiate β-cell apoptosis that is induced by ER stress (Fang *et al.*, 2014) or elevated concentrations of free fatty acid (Qin *et al.*, 2014), conditions that contribute critically to the development of β-cell insufficiency in type 2 diabetes (Cnop *et al.*, 2005). Additionally, pancreatic islets isolated from *Trib3*-deficient mice display reduced susceptibility to cytokine-induced β-cell death in an *ex vivo* model of the autoimmune β-cell destruction that occurs during the pathogenesis of type 1 diabetes (Humphrey *et al.*, 2014).

2.4.6.2. The effect of TRIB3 on diabetes complications

Diabetic kidney disease is a common complication of longstanding diabetes (Kumar *et al.*, 2015), and the role of *TRIB3* in the development of diabetic nephropathy has also been studied. *Trib3*$^{-/-}$ mice with diabetes induced by streptozotocin, a compound that causes pancreatic β-cell destruction, develop more severe kidney dysfunction than *Trib3*$^{+/+}$ mice, indicating a protective effect of TRIB3 in the diabetic kidney (Borsting *et al.*, 2014). This effect is thought to involve the attenuation of mTORC2-mediated Akt activation in the kidney by the binding of TRIB3 to the mTORC2 components mTOR and

RICTOR (Borsting *et al.*, 2014). On the other hand, studies involving the *in vivo* knockdown of *Trib3* in rats with either streptozotocin-induced diabetes or high-fat diet-induced insulin resistance indicate that TRIB3 supports the development of diabetic renal pathology (Ding *et al.*, 2014; Wang *et al.*, 2014). The divergent effects of TRIB3 may be due to the peculiarities of the different disease models and whether *Trib3* was silenced after the onset of disease or had been constitutively knocked out. As a gene implicated in insulin signaling, *Trib3* could also alter the underlying metabolic disorder that leads to the kidney pathology being investigated. Notably, in the study of diabetic nephropathy in mice with streptozotocin-induced diabetes, the level of hyperglycemia was similarly severe in *Trib3* knockout and wild type mice (Borsting *et al.*, 2014).

Diabetes also increases the risk of cardiovascular disease, and, in a mouse model of diabetic atherosclerosis, *in vivo* silencing of *Trib3* reduced the extent of atherosclerotic plaques and improved plaque stability (Wang *et al.*, 2012); therefore, *Trib3* potentially increases the risk of acute myocardial infarction. Further, following an experimental myocardial infarction, mice with cardiac muscle-specific overexpression of *Trib3* develop more extensive damage to the heart than wild type mice (Avery *et al.*, 2010). Pathological cardiac remodeling can involve cardiomyocyte loss due to excess mechanical stress, and *TRIB3* has been uncovered as a major contributor to stretch-induced cardiomyocyte apoptosis (Cheng *et al.*, 2015). Based on *in vivo* gene silencing experiments, *Trib3* is also implicated in the development of diabetic cardiomyopathy, a deterioration of heart function that develops as a complication of prolonged diabetes but is not explained by coronary artery disease (Ti *et al.*, 2011). The beneficial effects of *Trib3* silencing on diabetic atherosclerosis and diabetic cardiomyopathy may be mediated in part by improved metabolic status, as *Trib3* silencing also resulted in markedly improved control of blood glucose (Ti *et al.*, 2011; Wang *et al.*, 2012).

2.4.6.3. A common, non-synonymous TRIB3 variant associated with human diseases: the Q84R substitution

In human *TRIB3*, a non-synonymous single-nucleotide polymorphism that results in the substitution of glutamine at position 84 of the TRIB3 protein with arginine (Q84R) has been described (dbSNP ID: rs2295490, alleles: A/G on the forward strand of the chromosome and also on the sense strand of the gene; Prudente *et al.*, 2005). This genetic variation is relatively common, with the minor allele (G, corresponding to TRIB3 R84) having an overall frequency of 0.20 in a global sample of 2504 people from 26 populations (The 1000 Genomes Project, Phase 3, May 2013 call set; Auton *et al.*, 2015). The human minor allele is actually the ancestral variant, and the arginine encoded by it is conserved in human TRIB1 and TRIB2, mouse TRIB1, TRIB2 and TRIB3, and *Drosophila* Tribbles (Hegedus *et al.*, 2007).

Human TRIB3 R84 is considered a gain-of-function variant since it confers TRIB3 with a superior ability to bind Akt and inhibit insulin-stimulated Akt phosphorylation (*i.e.*, activation), compared to the TRIB3 Q84 variant (Andreozzi *et al.*, 2008; Formoso *et al.*, 2011; Liew *et al.*, 2010; Prudente *et al.*, 2005). Based on molecular modeling, the Q84R substitution is thought to cause structural differences in the Akt-interacting region (Andreozzi *et al.*, 2008). Additionally, the TRIB3 R84 protein demonstrates a prolonged half-life compared to TRIB3 Q84 (Liew *et al.*, 2010).

In human populations, the TRIB3 R84 variant is associated with a number of health issues, including reduced insulin sensitivity, impaired insulin secretion, type 2 diabetes, metabolic syndrome, abdominal obesity, hypertriglyceridemia, atherosclerosis, insulin resistance-related myocardial infarction, and type 2 diabetes-related kidney disease (Bacci *et al.*, 2013; De Cosmo *et al.*, 2007; Formoso *et al.*, 2011; Gong *et al.*, 2009; Liew *et al.*, 2010; Prudente *et al.*, 2005; Prudente *et al.*, 2009; Prudente *et al.*, 2010; Prudente *et al.*, 2013; Shi *et al.*, 2009; Zhang *et al.*, 2015). The TRIB3 Q84R polymorphism has not been examined in microarray-based large-scale genome-wide association studies of type 2 diabetes or related traits, since this particular polymorphism has not been included the experimental designs and is not linked to any of the analyzed polymorphisms, precluding allele inference (Bacci *et al.*, 2013; Liew *et al.*, 2010; Prudente *et al.*, 2010; Prudente *et al.*, 2013). Thus, the associations between TRIB3 position 84 variants and abnormalities of glucose homeostasis require further validation in large-scale studies in order to achieve ‘genome-wide significance’ (Prudente *et al.*, 2012).

3. AIMS OF THE PRESENT STUDY

In *Drosophila*, Tribbles is a developmentally important protein that inhibits mitosis. In mammals, the fruit fly *tribbles* gene has three homologs, *TRIB1*, *TRIB2* and *TRIB3*, which appear to have been repurposed over the course of evolution. *TRIB3* has acquired distinct expression characteristics, displaying mRNA upregulation in response to cellular stress in a wide variety of cell types, and although numerous roles associated with stressful conditions as well as normal physiology have already been attributed to *TRIB3*, there still remains much to be learned about the functions of the *TRIB3* gene.

The specific aims of this work are the following:

- to design and validate an experimental approach for comparing the abundance of human *TRIB3* mRNA variants,
- to characterize the composition of the human *TRIB3* mRNA population under normal and stressful conditions,
- to analyze the expression of *Trib3* in mouse bone marrow-derived mast cell (BMMC) cultures responding to mast cell biology-related signals (such as stimulation by the growth factor IL-3, sensitization by IgE and the induction of degranulation by antigen),
- to determine the capability of *Trib3*-deficient BMMCs to perform immunological functions (such as degranulation and activation-induced cytokine mRNA expression),
- to examine the expression of *Trib3* and the other *tribbles* homologs during the course of mouse brain development and in different regions of the adult mouse brain,
- to uncover if a lack of *Trib3* affects mouse behavioral responses that are dependent on the eIF2α–ATF4 pathway, which TRIB3 is known to inhibit in cell culture experiments,
- to elucidate the mechanism that leads to *TRIB3* upregulation in glucose-starved cells,
- to investigate the effect of TRIB3 on the transcriptional response to glucose deprivation, using genome-wide gene expression analysis, and
- to shed light on the mechanism how elevated *TRIB3* expression affects the viability of cells subjected to glucose-deficient conditions.

4. RESULTS AND DISCUSSION

4.1. Development of an assay system for the comparative quantification of human *TRIB3* mRNA isoforms (Ref. I)

A previous study by our group uncovered the existence of several human *TRIB3* mRNA isoforms, which are generated by the use of alternative transcription initiation sites and alternative splicing of the first exon (Örd and Örd, 2005). In all cases, the first *TRIB3* exon encompasses precisely the mRNA 5′-UTR and the second exon begins with the translation initiation codon (as described in section 2.3.5). As a result, the 5′-UTR sequence can vary completely between the different *TRIB3* mRNA isoforms, while the encoded TRIB3 protein is predicted to be identical in all cases (Örd and Örd, 2005). Since the properties of the 5′-UTR play a principal role in determining the rate of protein synthesis from an mRNA molecule, variation of the 5′-UTR can affect gene expression at the translational level (Hughes, 2006; Kozak, 2005). Additionally, alternative mRNA 5′-end regions may arise through the activation of alternative promoters and thereby signify a mode of transcriptional regulation (Hughes, 2006).

In order to achieve a better understanding of *TRIB3* gene expression regulation, a project was initiated to characterize the composition of the *TRIB3* mRNA pool in cells subjected to regular or stressful conditions. To enable this, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) system that allows the comparative quantification of the different *TRIB3* mRNA isoforms was designed and validated. In the assay system, template amplification is detected by SYBR Green I dye binding to double-stranded DNA, the PCR product. The five different splice variants of exon 1 (1A, 1B1, 1B2, 1B3 and 1B4) are quantified using RT-qPCR amplicons consisting of a splice variant-specific primer that binds to the exon 1–exon 2 junction and a common primer that binds to the invariant exon 2 sequence. Additionally, since the exon 1A transcription initiation sites span a region of several hundred base pairs, three amplicons were positioned along the exon 1A sequence to distinguish splice variant 1A 5′-UTRs with a minimal length of either 184, 283 or 343 nucleotides (sequence lengths are based on a *TRIB3* allele that contains two copies of the polymorphic 33-bp tandem repeat discussed in section 2.3.3.1). Finally, a primer pair targeting the *TRIB3* protein-coding region, which is common to all *TRIB3* mRNA isoforms, was used to acquire the total *TRIB3* mRNA level.

The specificity of each isoform-specific amplicon toward its target isoform was assessed by qPCR, using plasmids that contained the cDNA of either the target or non-target *TRIB3* mRNA variants as reaction templates. RT-qPCR results obtained with different amplicons are initially not directly comparable to each other, due to technical reasons arising from the qPCR step (such as different reaction amplification efficiencies for different primer pairs and differences in the amount of fluorescent dye bound per product molecule) and especially from the reverse transcription step (variable efficiency of reverse

transcription in different mRNA regions due to inefficient priming or mRNA secondary structures that inhibit reverse transcriptase progression) (Stahlberg *et al.*, 2004; Stangegaard *et al.*, 2006; Vandenbroucke *et al.*, 2001; Zhang and Byrne, 1999). Thus, several additional methodological steps were required to obtain RT-qPCR-based data that allows for the comparison of the abundances of the different *TRIB3* mRNA isoforms. Plasmid-based standard curves were used to correct for the differences originating from the qPCR step, and the reverse transcription efficiencies of the different *TRIB3* amplicon regions were determined by quantifying the reverse transcription of *in vitro*-synthesized full-length human *TRIB3* mRNA spiked into mouse total RNA.

4.2. Cellular stress alters the composition of the *TRIB3* mRNA population in human hepatoma cells, potentiating protein production (Ref. I)

HepG2 human hepatocellular carcinoma cells were selected to be the subject of the *TRIB3* mRNA isoform quantification, since these cells display readily detectable basal *TRIB3* mRNA expression and robust *TRIB3* induction in response to stress (Örd and Örd, 2005), and they have been used extensively as a model system for eIF2α–ATF4 pathway-based stress responses (for example, Shan *et al.*, 2010). Additionally, the liver is a major site of *TRIB3* expression in the body (discussed in section 2.3.1), and HepG2 cells have retained a considerable degree of hepatocyte metabolism and gene expression (Javitt, 1990; Tyakht *et al.*, 2014). To activate ATF4-mediated cellular stress responses, the cells were treated with arsenite, a known inducer of oxidative stress, protein misfolding and eIF2α phosphorylation (Bernstam and Nriagu, 2000; Brostrom *et al.*, 1996; Zhou *et al.*, 2008a).

The results reveal that in HepG2 cells incubated in normal growth medium, the predominant *TRIB3* mRNA splice variant is 1A, which constitutes slightly over 90% of the *TRIB3* mRNA population, and the remainder is mostly made up of 1B4 (8%), while the splice variants 1B1, 1B2 and 1B3 together account for less than 1% of the mRNA population. In response to arsenite treatment, the overall level of *TRIB3* mRNA is upregulated 23-fold. The splice variants 1A, 1B1, 1B2 and 1B3 are all induced by around 20-fold, while splice variant 1B4 is upregulated more than 50-fold. Thus, in arsenite-treated cells, splice variant 1A remains predominant and 1B1–1B3 remain marginal, but the prevalence of 1B4 increases substantially (to 15%) in stressed cells as a consequence of the more prominent induction of 1B4 compared to other splice variants.

The *TRIB3* C/EBP–ATF composite site, which mediates *TRIB3* gene induction in response to stress, is located in 33-bp tandem repeats that are situated 192 bp upstream of the exon 1A splice donor site. RT-qPCR results obtained with amplicons corresponding to different regions of exon 1A uncovered that under normal growth conditions, around half of the 1A mRNAs are generated using transcription start sites located upstream of the 33-bp repeat region, while

only around 10% are initiated downstream of the 33-bp repeats. These proportions are drastically altered in response to arsenite stress: the percentage of 1A mRNAs extending upstream of the 33-bp repeats is diminished (to approximately 3%) and the percentage of 1A mRNAs initiated downstream of the 33-bp repeats rises to above 80%. Thus, the upregulation of *TRIB3* mRNA level in response to stress is mainly achieved by the production of splice variant 1A mRNAs with shortened 5′-UTRs, due to the preferential use of transcription start sites located downstream of the 33-bp repeat regions, while splice variant 1A mRNAs with long 5′-UTRs initiated upstream of the 33-bp repeats contribute significantly to the *TRIB3* mRNA pool under basal conditions.

Results obtained by D. Örd (presented in Ref. I) reveal that splice variant 1A mRNAs with short 5′-UTRs are translated two-fold more efficiently in HepG2 cells than those with long 5′-UTRs. Strong mRNA secondary structures formed from GC-rich sequences in 5′-UTRs impair translation (Babendure *et al.*, 2006; Kozak, 1989; Pickering and Willis, 2005), and both long and short forms of human *TRIB3* splice variant 1A mRNA 5′-UTRs display nearly 80% GC content, which is above the human average of around 60% (Kalari *et al.*, 2006; Pesole *et al.*, 2001). Additionally, the long forms of *TRIB3* splice variant 1A 5′-UTRs contain two short ORFs that are situated upstream of the *TRIB3* protein-coding ORF. These 5′-UTR elements, termed ‘upstream ORFs’ (uORFs), are present in more than 40% of human mRNAs (Peri and Pandey, 2001) and may inhibit the translation of the protein-coding ORF by directing ribosomes to initiate translation from the uORF start codon, provided that the uORF start codon is in a favorable nucleotide context for translation initiation (Kozak, 2001; Wang and Rothnagel, 2004). The initiation codon of one of the *TRIB3* uORFs is in an optimal context for translation initiation (Kozak, 1986; Kozak, 1987), and further experiments performed by D. Örd (Ref. I) uncovered that the inclusion of this uORF underlies the different translational efficiencies of the long and short forms of *TRIB3* splice variant 1A mRNAs.

Taken together, the results of Ref. I indicate that transcriptional and translational mechanisms act in concert to facilitate the upregulation of *TRIB3* in response to arsenite-induced cellular stress in HepG2 cells. In arsenite-treated cells, *TRIB3* transcription initiation sites are shifted downstream compared to untreated cells, leading to an increased proportion of *TRIB3* mRNAs with splice variant 1B4 and short 1A 5′-UTRs. As a result of this shift, stress-induced *TRIB3* transcription is predominantly initiated downstream of the 33-bp tandem repeats which contain the stress-responsive C/EBP–ATF composite sites (described in section 2.3.3.1).

Variation of the first exon is often associated with alternative promoter usage (for example, Holthuizen *et al.*, 1993; Hughes and Brady, 2005; Martineau *et al.*, 2004; Sobczak and Krzyzosiak, 2002), and the distribution of the *TRIB3* transcription start sites over a range of roughly 500 bp is compatible with the concept of multiple core promoters, since human core promoters typically utilize transcription initiation sites spread over 50–100 bp (Sandelin *et al.*, 2007). As discussed in section 2.3.3.1, C/EBP–ATF composite sites themselves

are flexible regarding positional requirements and satisfy the criteria for classification as enhancers. Arsenite-treated HepG2 cells preferentially induce *TRIB3* splice variant 1A mRNAs that contain a truncated 5′-UTR, which boosts protein synthesis due to the lack of an inhibitory uORF. Elimination of an inhibitory uORF by 5′-UTR truncation has also been described for some other genes (Blaschke *et al.*, 2003; Phelps *et al.*, 1998).

Future studies quantifying *TRIB3* mRNA isoforms are warranted in order to uncover the cell- and stress-type specificity of the *TRIB3* mRNA variant profile. In addition to differentially containing uORFs, *TRIB3* 5′-UTRs are GC-rich and presumably impede translation also by forming secondary structures. It may be worth investigating whether *TRIB3* mRNAs are subject to context-dependent derepression by increased translation initiation factor eIF4E activity, which alleviates the inhibitory effects of GC-rich 5′-UTRs (Koromilas *et al.*, 1992; Nikolcheva *et al.*, 2002; Rosenwald, 2004).

4.3. *Trib3* expression in mouse bone marrow-derived mast cells (BMMCs) is upregulated by interleukin 3 (IL-3), a major mast cell growth factor (Ref. II)

Mast cells are tissue-resident immune cells that serve as the principal effector cells for immunoglobulin E (IgE)-mediated acquired immune responses and also participate in innate immune responses (Galli *et al.*, 2005). Mast cells are critical for providing host defense against helminthic parasites in the intestine; however, they are also central for the manifestation of allergic disorders (Abraham and St. John, 2010; Kumar *et al.*, 2015). Mast cells are characterized by the presence of a large number of cytoplasmic granules that are filled with pro-inflammatory mediators such as histamine (Galli *et al.*, 2005). Upon activation, mast cells rapidly secrete the preformed inflammatory mediators stored in their granules (‘degranulation’) and subsequently proceed to release newly-formed lipid-derived mediators (such as prostaglandins and leukotrienes), cytokines (including TNF, IL-1β and IL-6) and chemokines (Abraham and St. John, 2010; Galli *et al.*, 2005).

Developmentally, mast cells are hematopoietic cells and thus originate from bone marrow; however, mature mast cells ordinarily do not circulate in the blood, even though mast cells are widely distributed in tissues (Ribatti and Crivellato, 2014). Rather, mast cell precursors circulate in low numbers and enter peripheral tissues, where they complete their differentiation and remain as long-lived cells (Abraham and St. John, 2010). Chief factors driving mast cell proliferation and maturation in mice are stem cell factor and IL-3 (Ribatti and Crivellato, 2014). The injection of IL-3 into mice dramatically increases the number of mast cells (Abe *et al.*, 1988; Metcalf *et al.*, 1986), and IL-3 knockout mice display a profound attenuation of mast cell hyperplasia in response to intestinal nematode infection, along with delayed clearance of the infection (Lantz *et al.*, 1998). *In vitro*, culturing mouse bone marrow cells in the presence of IL-3 can be used to produce an ample and high-purity supply of mast cells

(Jensen *et al.*, 2006). These cells, termed BMMCs, are widely used to study mast cell function *in vitro* (Jensen *et al.*, 2006) and are even suitable for reconstituting mast cell populations in mice genetically lacking mast cells (Grimbaldeston *et al.*, 2005; Wolters *et al.*, 2005).

TRIB3 has previously been implicated in mediating the response to erythropoietin, the principal cytokine controlling erythrocyte production, in erythroid progenitors (Sathyanarayana *et al.*, 2008). Additionally, high *Trib3* expression has been noted in bone marrow (Kiss-Toth *et al.*, 2004), and roles in normal hematopoiesis and/or hematopoietic malignancy have been uncovered for *Trib1* and *Trib2* (Stein *et al.*, 2015). While TRIB3 has been implicated in the regulation of inflammatory processes (discussed in section 2.4.5), little is known about the importance of TRIB3 in immune cells, including mast cells.

In Ref. II, BMMCs derived from wild type mice were used to study whether *Trib3* expression is modulated by treatments that generate mast cell-specific responses. Mast cells are primed to react to a specific antigen ('sensitized') by the binding of IgE to the high-affinity IgE receptor (Abraham and St. John, 2010). IgE binding also enhances mast cell survival (Asai *et al.*, 2001). Mast cells are activated (that is, triggered to secrete inflammatory mediators) when a multivalent antigen concurrently binds to multiple IgE molecules, resulting in the cross-linking of IgE receptor molecules (Abraham and St. John, 2010). In BMMCs, the *Trib3* mRNA level is not altered in response to sensitization, and BMMC activation leads to a modest decrease in *Trib3* expression.

After Ref. II had been submitted, Kuo *et al.* (2012) published gene expression microarray results showing that *Trib3* is prominently upregulated in response to IgE sensitization in RBL-2H3 rat basophilic leukemia cells expressing human chemokine receptor 1 (RBL-CCR1) (the effect of IgE receptor cross-linking on *Trib3* expression was not analyzed). They also present data that in RBL-CCR1 cells sensitized with IgE, *Trib3* inhibits cytokine production (Kuo *et al.*, 2012). However, the effect of *Trib3* on the response to IgE receptor cross-linking (*i.e.*, activation), which dramatically increases cytokine production beyond the level generated by IgE treatment alone (Koranteng *et al.*, 2004), was not examined (Kuo *et al.*, 2012). RBL-2H3 cells have some mast cell-like and some basophil-like characteristics, and have undergone malignant transformation, including constitutive activation of growth factor receptor signaling (Passante and Frankish, 2009; Passante *et al.*, 2009; Tsujimura *et al.*, 1995). For these reasons, they are considered problematic as a model of primary mast cell physiology (Jensen *et al.*, 2006; Passante and Frankish, 2009). Indeed, Kuo *et al.* (2012) compare their RBL-CCR1 IgE sensitization gene expression microarray results to results obtained using primary human mast cells (analyzed in Jayapal *et al.*, 2006) and note that there is no overlap between the two cell types in the genes that are induced at least two-fold by IgE sensitization (Kuo *et al.*, 2012).

BMMCs are dependent of IL-3 for proliferation and survival, and, as demonstrated in Ref. II, the removal of IL-3 from the growth medium causes a decline in *Trib3* expression, which is reversible by the re-addition of IL-3. Since IL-3

can elicit a wide range of gene expression alterations by affecting mRNA turnover (Ernst *et al.*, 2009), the level of unspliced *Trib3* precursor mRNA was quantified as a proxy for the rate of gene transcription (Lipson and Baserga, 1989). The results reveal that *Trib3* pre-mRNA is also induced by IL-3, indicating regulation at the transcriptional level. Thus, *Trib3* gene activation is positively regulated in BMMCs by IL-3, their requisite cytokine.

There are few previous reports of TRIB3 regulation by hematopoietic growth factors. In an erythroleukemia cell line, *TRIB3* was found to be down-regulated in response to the withdrawal of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lin *et al.*, 2007), a cytokine which shares a common receptor with IL-3 (Martinez-Moczygemba and Huston, 2003). Downstream of its receptor, IL-3 modulates at least three major signaling pathways: Jak/STAT, MAPK and PI3K (Martinez-Moczygemba and Huston, 2003). Of these pathways, positive regulation of *Trib3* has been associated with the activity of STAT5 downstream of erythropoietin and PI3K downstream of insulin (Ding *et al.*, 2008; Sathyanarayana *et al.*, 2008). Both STAT5 and PI3K are known to be critical for IL-3 signaling and functional effect in BMMCs (Ali *et al.*, 2004; Shelburne *et al.*, 2003), and could prove to convey the effect of IL-3 on the *Trib3* gene.

Besides *Trib3*, several other cellular stress-inducible genes, such as *Atf3*, *Atf5* and *Xbp1*, are also positively regulated by IL-3 in cells of hematopoietic origin (Gilchrist *et al.*, 2010; Kurata *et al.*, 2011; Persengiev *et al.*, 2002), and the upregulation of these genes by IL-3 could indicate that stress response pathways are utilized to maintain the physiology of cytokine-stimulated cells. Particular immune cell functions are certainly known to require the integrated stress response pathway (Mielke *et al.*, 2011; Scheu *et al.*, 2006), for which *Trib3* (as discussed in section 2.3.3) as well as *Atf3* and *Atf5* are downstream targets (Deval *et al.*, 2009; Pan *et al.*, 2007; Shan *et al.*, 2009; Shan *et al.*, 2010).

4.4. Lack of *Trib3* impairs BMMC immunological functions (Ref. II)

Our group has generated a novel mouse line carrying a targeted deletion of *Trib3* (first described in Ref. II). In these mice, the entire *Trib3* protein-coding region, which spans from exon 2 to exon 4, has been eliminated, thereby precluding the production of truncated protein forms. The absence of *Trib3* has been confirmed using several approaches (Refs. II and III). In line with the *Trib3* knockout mouse line derived independently by Okamoto *et al.* (2007), homozygous *Trib3* null individuals from our mouse line are viable and physically grossly normal.

To study the importance of *Trib3* in mast cells, BMMCs were derived from *Trib3*-deficient mouse bone marrow. The development of mast cells from bone marrow cell culture can be monitored by periodically examining the cells for

the presence of mature mast cell surface markers using flow cytometry (Jensen *et al.*, 2006). Experiments performed by D. Örd (Ref. II) indicate that in the absence of *Trib3*, the kinetics of BMMC differentiation in IL-3-supplemented medium are unaltered, and that the mature mast cell populations produced from *Trib3*$^{-/-}$ bone marrow cultures are similar to *Trib3*$^{+/+}$ cultures in cell quantity, purity and IgE receptor expression level. To evaluate the functional capabilities of the BMMCs, the cells were sensitized using IgE that is specific to dinitrophenyl (a synthetic antigen) and subsequently activated with the corresponding multivalent antigen, dinitrophenyl-conjugated bovine serum albumin. Following activation, the extent of BMMC degranulation was determined by measuring the release of β-hexosaminidase, an enzyme present in mast cell granules and involved in anti-bacterial defense (Fukuishi *et al.*, 2014; Schwartz *et al.*, 1979), and the induction of cytokine production was quantified by monitoring the mRNA levels of TNF and IL-6, two important cytokines secreted by activated mast cells (Abraham and St. John, 2010).

The results reveal that *Trib3*-deficient BMMCs display an impairment of degranulation as well as cytokine gene upregulation in response to IgE-mediated activation. Their ability to degranulate is compromised over a wide range of antigen concentration, and also when BMMC activation is induced by pharmacological means that bypass the IgE receptor altogether.

In light of these results, *in vivo* assessment of dermal mast cell activity was carried out using a passive cutaneous anaphylaxis assay, which measures dye extravasation into tissue as a result of vasodilation caused by mast cell degranulation. In this model, there is no apparent difference between *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice. It is possible that compensatory mechanisms are acting *in vivo* to mask the effect of *Trib3* deficiency in skin mast cells or that the role of *Trib3* is limited to certain mast cell subtypes. There are two major phenotypically distinct types of mast cells in the mouse: connective tissue-type mast cells, such as those in the skin, and mucosal-type mast cells, such as those in the intestinal mucosa (Gurish and Austen, 2012). The mast cells that arise from culturing mouse bone marrow cells in medium supplemented with IL-3 largely resemble the mucosal type (Gurish *et al.*, 1992; Jensen *et al.*, 2006), highlighting that an *in vivo* examination of mucosal mast cells in *Trib3* knockout mice may also be warranted.

Currently, it remains unknown how *Trib3* deletion leads to defects in BMMC function. Recent reports suggest that in some situations, TRIB3 supports the activation of NF-κB (Fang *et al.*, 2014; Yu *et al.*, 2015). In BMMCs, NF-κB activation mediates the induction of the cytokines TNF and IL-6 in response to IgE receptor cross-linking; however, NF-κB activity is dispensable for BMMC degranulation, which *Trib3* deletion also affects in addition to cytokine induction (Marquardt and Walker, 2000; Peng *et al.*, 2005). BMMCs appear to require the induction of autophagy for degranulation (Ushio *et al.*, 2011), and, in glioma cells, TRIB3 can potentiate autophagy by reducing mTORC1 activity (Salazar *et al.*, 2009a), thereby providing a possible mechanism for the degranulation defect resulting from *Trib3* gene disruption.

Note that cytokine induction is unaffected in autophagy-defective BMMCs (Ushio *et al.*, 2011). Recent reports have uncovered that TRIB3 binds to RICTOR and possibly decreases its function (Borsting *et al.*, 2014), and that endogenous RICTOR is a suppressor of mast cell activation (Smrz *et al.*, 2014). Modulation of RICTOR by TRIB3 could partly, but not entirely, explain the potentiation of BMMC activation by *Trib3*, since RICTOR acts in an IgE receptor-specific manner (Smrz *et al.*, 2014), while the effect of *Trib3* is also evident when proximal IgE receptor signaling is bypassed.

The quite broad impairment of immunological functions in *Trib3* knockout BMMCs is consistent with a signaling defect that is situated relatively downstream in the mast cell activation signaling cascade (Baba *et al.*, 2008; Nechushtan *et al.*, 2000). Growth factors can assert even broader effects on cells, and the major growth factor for BMMCs, IL-3, is known to affect not only mast cell development, proliferation and survival but also immunological responses (Coleman *et al.*, 1993; Gebhardt *et al.*, 2002; Mekori *et al.*, 1993; Minks *et al.*, 1992). Experiments performed by D. Örd and included in Ref. II show that *Trib3*-deficient BMMCs are more sensitive to IL-3 deprivation than wild type BMMCs, displaying delayed cell cycle resumption following short-term transient IL-3 deficiency and accelerated cell death during prolonged IL-3 withdrawal. Thus, $Trib3^{-/-}$ BMMCs appear more susceptible to inadequate growth factor action than $Trib3^{+/+}$ BMMCs, which could also underlie the reduced responsiveness of *Trib3*-deficient BMMCs to immunological stimulation. If this is the case, *Trib3* may be induced by IL-3 (described in section 4.3) in order to potentiate certain aspects of IL-3 signaling, such as the preservation of mast cell function and viability. Therefore, the effector pathways downstream of the IL-3 receptor warrant further investigation in order to elucidate the signals leading to BMMC *Trib3* expression as well as to determine the contribution of TRIB3 to IL-3 signaling.

4.5. *Trib3* expression increases during mouse brain development and $Trib3^{-/-}$ mice display enlarged lateral ventricles in the brain (Ref. III)

The sole *tribbles* gene in *Drosophila* encodes a developmental regulatory protein (as discussed in section 2.1); however, the knockout mice for the mammalian *tribbles* genes appear physically grossly normal (discussed in section 2.4.3.1). *Trib3* was initially isolated from neuronal cell cultures (Mayumi-Matsuda *et al.*, 1999; Örd and Örd, 2003) and was subsequently found to directly inhibit ATF4 (Bowers *et al.*, 2003; Örd and Örd, 2003). In addition to playing a central role in the mammalian cellular stress response, ATF4 (alias CREB2, cAMP response element-binding protein 2) has also been described as a suppressor of CREB-dependent memory formation, based on work originally done in the sea slug *Aplysia californica* (Bartsch *et al.*, 1995; Karpinski *et al.*, 1992). Therefore, the characterization of the role of *Trib3* in the brain was initiated.

First, to study the course of *tribbles* homolog gene expression during brain development, RT-qPCR was used to analyze *Trib1*, *Trib2* and *Trib3* mRNA levels in the mouse brain (sans cerebellum) from embryonic day (E) 14 to post-natal day (P) 4. The typical gestation period for laboratory mice is 19–20 days (Hedrich, 2004). The results show that *Trib3* expression increases steadily in the mouse brain from E14 to E18, amounting in total to an upregulation of approximately 4-fold. After birth (at P0), the *Trib3* mRNA level appears to decrease somewhat; however, the expression level recovers by P2, reaching nearly 6-fold above the E14 level, and remains high at P4. In comparison, the expression of both *Trib1* and *Trib2* remains relatively steady during brain development, with expression levels varying less than 2-fold over the entire period from E14 to P4. Additionally, it seems that there is no compensatory upregulation of *Trib1* or *Trib2* expression in the brain in response to a lack of *Trib3*, as an analysis of P3 mouse brains revealed no differences in *Trib1* or *Trib2* expression levels between $Trib3^{+/+}$, $Trib3^{+/-}$ and $Trib3^{-/-}$ mice. Based on these results, *Trib3* deficiency could affect mouse brain development.

Since nerve growth factor deprivation induces *Trib3* (Angelastro *et al.*, 2000; Mayumi-Matsuda *et al.*, 1999; Zareen *et al.*, 2013), the elevation of *Trib3* mRNA level in the developing brain could originate from excess neurons that are suffering from neurotrophic factor deficiency and will eventually undergo apoptosis in order to be removed, a characteristic feature of vertebrate brain development (Blaschke *et al.*, 1996; Oppenheim, 1991). In addition to neuronal cells, microglial cells can also express *Trib3* (Juknat et al., 2012; Juknat et al., 2013). Thus, the cell type and circumstances generating *Trib3* expression in the developing brain await further investigation.

An examination of adult *Trib3* knockout mouse brain morphology, performed by K. Lilleväli and T. Tekko and included in Ref. III, revealed no apparent alterations to the gross morphology of prominent brain structures, except for a tendency towards larger lateral ventricle size in *Trib3*-deficient individuals. This trend was subjected to further investigation by measuring the lateral ventricle area from coronal sections of $Trib3^{+/+}$ and $Trib3^{-/-}$ mouse brains. Two independent groups of $Trib3^{+/+}$ and $Trib3^{-/-}$ mice were studied: an adult group, consisting of individuals approximately 6 months of age, and a juvenile (P9) group. The results demonstrate that in both groups, lateral ventricle size is significantly increased in *Trib3*-deficient individuals (by 1.5-fold in adults and 2-fold in juveniles). This suggests that *Trib3* affects the ventricular system of the brain but not other major anatomical structures.

The ventricles of the brain are a network of four chambers that are filled with cerebrospinal fluid, and the two lateral ventricles, which are the largest, form arc-shaped chambers in either cerebral hemisphere (Saladin, 2003). The interior surfaces of the ventricles are responsible for producing cerebrospinal fluid, which serves to provide neutral buoyancy and basic protection for the brain (Saladin, 2003). Although lateral ventricle size in humans increases with age and is linked to age-related brain disorders (Skullerud, 1985), it is also quite

variable among healthy, non-elderly people (Allen *et al.*, 2002). Similarly, brain ventricle size varies between different strains of laboratory mice, and the C57BL/6 strain, which the *Trib3* knockout mouse used in Ref. III is based upon, exhibits a moderate lateral ventricle size (Hino *et al.*, 2009). Extreme enlargement of ventricles is seen in hydrocephalus, which is caused by excessive cerebrospinal fluid accumulation (Kumar *et al.*, 2015). The occurrence of congenital hydrocephalus is increased in the C57BL/6J strain of mice compared to several other strains, but the incidence is still low (0.029% according to data available at: www.jax.org/news-and-insights/2003/july/hydrocephalus-in-laboratory-mice, accessed 2015-11-24). No young *Trib3* knockout mice have presented with overt hydrocephalus, though the sample size is insufficient to rule out a minor increase in susceptibility (data not shown). In the future, it could be worthwhile to study whether aging-related ventricular expansion is affected by *Trib3* deficiency.

4.6. Amino acid-imbalanced diet induces *Trib3* in the mouse brain but *Trib3* is not essential for the aversive reaction to such diet (Ref. III)

In the mammalian brain, the eIF2α–ATF4 pathway is implicated in several behavioral processes. Essential amino acids (EAAs) are the amino acids that must be obtained from the diet because they cannot be synthesized *de novo* by the body (Saladin, 2003). Many foods contain EAAs in suboptimal proportions, and omnivores have evolved a mechanism to detect if a food source provides an inadequate amount of one or more EAAs, and they try to correct the imbalance by consuming a combination of different foods (Gietzen *et al.*, 2007). In a laboratory setting, this effect is evident when mice or rats are presented a diet that lacks a single EAA but is otherwise nutritionally complete; the animals will limit their intake of such a diet and search for alternative dietary sources (Gietzen *et al.*, 2007). This innate reaction takes place already during the first meal and is dependent on the sensing of serum amino acid levels by a specific region of the brain, the anterior piriform cortex (APC), but does not depend on taste, smell or gastrointestinal signals (Gietzen *et al.*, 2007).

At the molecular level, the eIF2α kinase GCN2 senses the amino acid deficit through the resulting accumulation of uncharged tRNA molecules and proceeds to phosphorylate eIF2α in the APC (Hao *et al.*, 2005; Koehnle *et al.*, 2004; Maurin *et al.*, 2005). Crucially, mice with a deletion of *Gcn2* fail to reject the EAA-insufficient diet; however, processes taking place downstream of eIF2α phosphorylation in this context are not well described (Hao *et al.*, 2005; Maurin *et al.*, 2005). Since TRIB3 is able to provide negative feedback of the eIF2α–ATF4 pathway in EAA-deprived cell cultures (Jousse *et al.*, 2007), experiments were carried out to determine if *Trib3* participates in the APC-dependent aversive reaction to EAA-insufficient diet.

To determine the expression level of *Trib3* in different regions of the adult brain, and whether *Trib3* is induced in response to an EAA-deficient diet, animals accustomed to a nutritionally complete synthetic diet were presented with either the same complete diet or a corresponding leucine-free diet for 6 h, and, subsequently, *Trib3* expression was analyzed from excised brain tissue using RT-qPCR. The results show that in complete diet-fed mice, the APC, the frontal cerebral cortex and the hippocampus have very similar levels of *Trib3* expression. However, in mice fed the leucine-deficient diet, *Trib3* expression is upregulated 3-fold specifically in the APC. The cerebellum displays a high level of *Trib3* expression, which is unaffected by diet type. For comparison, the expression levels of *Trib1* and *Trib2* were also analyzed. *Trib1* and *Trib2* display relatively uniform levels of expression across the examined brain regions, and neither of them is affected by diet composition in any of the brain regions. Thus, *Trib3* has the most distinctive adult brain gene expression pattern of the three *tribbles* genes, with high basal expression in the cerebellum and diet-responsive expression in the APC.

Recently, the Allen Brain Atlas project published an extensive gene expression analysis of the human brain, providing genome-wide gene expression data at an exceptionally high anatomical resolution, with approximately 500 samples collected per brain hemisphere per donor (data available at: www.brain-map.org, accessed 2015-12-07; Hawrylycz *et al.*, 2015). To evaluate the biological importance of a gene for brain processes, Hawrylycz *et al.* (2015) analyzed the extent of reproducible (conserved) structural patterning of gene expression across brain regions, a metric which ranked *TRIB3* among the top 15% most relevant genes. The expression pattern of *TRIB3* in the human brain is characterized by a high level of expression in the cortex of the cerebellum, and a moderate and uniform expression level across different regions of the cerebral cortex—trends which are in excellent agreement with the mouse brain data obtained and presented in Ref. III. Additionally, the Allen Brain Atlas data reveals exceptionally low *TRIB3* expression in the corpus callosum, the largest white matter structure in the human brain. Since the corpus callosum is rich in glial cells, while grey matter structures are rich in neuron bodies, the expression pattern of *TRIB3* points to it being expressed in neuronal cells as opposed to glial cells.

To assess the importance of *Trib3* for the rejection of EAA-deficient diet, a method adapted from Maurin *et al.* (2005) was used. Prior to the experiment, adult $Trib3^{+/+}$ and $Trib3^{-/-}$ mice were trained to a cycle of 12 h night-time fasting–12 h day-time feeding with synthetic diet. During the experiment, the intake of either synthetic leucine-free diet or control (nutritionally complete) synthetic diet was measured for each animal at the 0.5, 1, 2, 4 and 12 h time-points. The results reveal that both genotypes exhibit a similar degree of EAA-deficient diet rejection, consuming approximately 30% less of the leucine-free diet than the control diet. The self-imposed food intake restriction is apparent already at the earliest time-point, 30 minutes, and is sustained at a similar level throughout the feeding period. The extent of the food intake decrease is similar to that

observed by Maurin *et al.* (2005) for their wild type mice. To assure that the food consumption data from *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice are comparable, their baseline body weights and the amount of weight lost during overnight fasting were also measured, revealing that these traits are not altered by *Trib3*-deficiency. Hence, it appears that although an EAA-insufficient diet markedly induces *Trib3* in the mouse APC, the location of the brain's chemosensor for EAA deficiency, a lack of *Trib3* is inconsequential for the avoidance of EAA-insufficient diet.

It seems probable that the induction of *Trib3* in the APC during EAA deficiency is mediated by C/EBP–ATF composite site-driven transcription downstream of the GCN2–eIF2α–ATF4 pathway, since the phosphorylation of eIF2α in EAA-deficient APC has been established (Hao *et al.*, 2005; Maurin *et al.*, 2005) and eIF2α phosphorylation-mediated *Trib3* upregulation has been verified in several other types of cells (described in section 2.3). Notably, there is little previous knowledge concerning the transcriptional responses of the APC to EAA-incomplete feeding, and it would be interesting to perform a broader, transcriptomic analysis. Functionally, aside from TRIB3, negative feedback regulation of the eIF2α–ATF4 pathway is also known to be provided by GADD34, a phosphatase regulatory subunit which directs eIF2α dephosphorylation (Ma and Hendershot, 2003). Currently, it cannot be excluded that alternative mechanisms of limiting eIF2α–ATF4 pathway activity are masking the effect of *Trib3* deletion, thereby enabling normal aversive behavior towards EAA-insufficient diet in *Trib3*$^{-/-}$ mice.

4.7. *Trib3* is dispensable for long-term spatial memory and fear conditioning (Ref. III)

In addition to the sensing of amino acid deficiency in the APC region of the brain, the eIF2α–ATF4 pathway is also known to be involved for other cognitive processes, particularly hippocampus-dependent long-term memory formation, such as spatial memory. Behavioral training reduces eIF2α phosphorylation in the hippocampus, and, in mouse models with decreased hippocampal phospho-eIF2α, ATF4 is downregulated and the threshold for long-term spatial memory formation is lowered (Costa-Mattioli *et al.*, 2005; Costa-Mattioli *et al.*, 2007). Notably, increasing hippocampal eIF2α phosphorylation to an intermediate level that induces ATF4 but does not repress general translation is sufficient to impair hippocampus-dependent learning (Jiang *et al.*, 2010). These results indicate that ATF4 activity in the hippocampus negatively regulates long-term spatial memory, which suggests the possibility that endogenous regulators of ATF4 activity, such as TRIB3 (which is expressed in the hippocampus, as noted in section 4.6), could influence the response to behavioral training.

To examine the effect of *Trib3* deletion on hippocampus-dependent spatial learning and memory, *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice were compared in the Morris water maze experiment, a swimming-based navigation task where mice learn to

escape from the water by climbing onto a hidden platform located in a particular position in the pool (Morris *et al.*, 1982). Multiple training sessions are performed on consecutive days, and the time the mice take to reach the platform (the 'escape latency') on each training event is monitored (Morris *et al.*, 1982). The results reveal that the escape latencies of *Trib3*-deficient mice and wild type mice are similar across the entire four-day course of the experiment, with both genotypes demonstrating good learning ability. To further assess learning, a 'probe trial' was performed subsequently by removing the platform from the pool and monitoring the swimming trajectory of the mouse for one minute. In this test, both genotypes exhibited a similar, strong preference for the region of the pool that previously contained the hidden platform. Performance in the Morris water maze can be confounded by differences in locomotor ability, and the cerebellum, which coordinates muscular activity, expresses a relatively high level of *Trib3* mRNA (as noted in section 4.6). An analysis of the swimming speeds of $Trib3^{+/+}$ and $Trib3^{-/-}$ mice indicates that *Trib3*-deficiency does not compromise locomotor activity. Thus, the Morris water maze results demonstrate that a lack of *Trib3* does not significantly alter spatial learning ability in mice.

After the mice have been subjected to the basic Morris water maze task described above, the platform can be repositioned and the mice can be challenged to relearn the location of the platform ('reversal learning'), a test of behavioral flexibility (Vorhees and Williams, 2006). Interestingly, forebrain-specific deletion of the eIF2α kinase PERK, which results in decreased ATF4 abundance, does not affect standard Morris water maze learning but impedes reversal learning (Trinh *et al.*, 2012). Therefore, $Trib3^{+/+}$ and $Trib3^{-/-}$ mice were also subjected to the reversal learning procedure following the main Morris water maze experiment. However, neither the reversal training escape latencies nor the reversal probe trial results revealed any significant differences between the genotypes.

The eIF2α phosphorylation pathway is also involved in long-term fear memory (Costa-Mattioli *et al.*, 2005; Costa-Mattioli *et al.*, 2007; Zhu *et al.*, 2011). Fear memory is commonly studied by 'fear conditioning': evaluating how readily animals learn to associate an unpleasant stimulus (*e.g.*, an electric shock) with a neutral stimulus or context (*e.g.*, an auditory tone or a particular chamber) (LeDoux, 2000). Fear conditioning requires the amygdala, and contextual fear conditioning additionally requires the hippocampus, while auditory fear conditioning does not (LeDoux, 2000). To study the role of *Trib3* in fear memory, auditory and contextual fear conditioning experiments were performed with $Trib3^{+/+}$ and $Trib3^{-/-}$ mice, using the incidence of freezing behavior (immobility) to measure conditioned fear. For both types of fear conditioning, the results show a normal fear reaction in *Trib3*-deficient mice, suggesting that *Trib3* does not affect long-term fear memory.

It is an unexpected finding that the deletion of *Trib3* does not affect long-term spatial memory, spatial relearning or fear conditioning, a set of behavioral processes for which the involvement of eIF2α pathway activity modulation has

been previously established (Costa-Mattioli *et al.*, 2005; Costa-Mattioli *et al.*, 2007; Jiang *et al.*, 2010; Trinh *et al.*, 2012; Zhu *et al.*, 2011). A further eIF2α pathway-dependent cognitive function, the ability to sense amino acid imbalance in food, was also unaffected by a lack of *Trib3* (as discussed in section 4.6). These behavioral paradigms address various regions of the brain, including the hippocampus, the amgydala and the APC.

It is possible that in the brain, during a normal physiological state, TRIB3 is not a sufficiently potent regulator of the eIF2α–ATF4 pathway to affect behavioral responses. Moreover, the role of phospho-eIF2α and ATF4 in long-term memory also appears to be more complex than initially thought, as several recent reports suggest positive instead of negative regulation of long-term memory by ATF4, alluding that the intricacies of the genetic models and experimental protocols used in different studies may play a significant role (Ill-Raga *et al.*, 2013; Pasini *et al.*, 2015; Wei *et al.*, 2012). Unfortunately, constitutive *Atf4* knockout mice are unsuitable for many behavioral experiments, since they are effectively blind, have bone defects and display decreased body size (Hettmann *et al.*, 2000; Masuoka and Townes, 2002; Tanaka *et al.*, 1998; Yang *et al.*, 2004).

In addition to the behavioral data reported in the current work, the International Mouse Phenotyping Consortium has recently released data on the performance of *Trib3* knockout mice in a different set of behavioral experiments, which are not centered on eIF2α pathway-dependent behavioral paradigms, and reports that no significant genotype effects were found (data available at: www.mousephenotype.org, accessed 2015-10-29, database release 3.4; Koscielny *et al.*, 2014). Still, *Trib3* may be important for behavioral responses that are currently unexplored or during the presence of aggravating circumstances, such as aging, nutritional deficiencies or diseases.

4.8. ER stress-mediated ATF4 induction leads to *TRIB3* upregulation in response to glucose deprivation (Ref. IV)

Glucose deficiency is a cell death-inducing stress that may occur in the context of several diseases. For instance, in a mouse model of stroke, the concentration of glucose in the infarction core region decreases to more than 10-fold below baseline (Kiewert *et al.*, 2010). A glucose-deficient microenvironment is also characteristic to the interior regions of solid tumors, due to inefficient tumor vasculature, excessive cell proliferation and the abnormal metabolism of transformed cells (Fukumura *et al.*, 2010; Gillies *et al.*, 2008; Hirayama *et al.*, 2009). For example, the glucose level in human colon cancer tissue is on average 10-fold below that of normal colon tissue, while the amino acids levels are comparable between tumor and normal tissues or even elevated in the tumor tissue (Hirayama *et al.*, 2009). Malignant cells display a propensity to utilize glucose at a high rate and to metabolize it by aerobic glycolysis (Gillies *et al.*, 2008; Hanahan and Weinberg, 2011). This metabolic preference renders cancer

cells susceptible to compounds which inhibit glycolysis and thereby effectively evoke glucose deficiency (El Mjiyad *et al.*, 2011). Therefore, the cellular stress response to glucose deficiency is potentially important for the progression of acute ischemic diseases, tumor growth and anti-cancer therapy.

Since *TRIB3* is cellular stress-associated regulatory protein (2.4.1) that is overexpressed in many types of human tumors and linked to cancer patient prognosis (2.4.4.1), an investigation into the role of TRIB3 during glucose deprivation was initiated. HEK293-derived cells were selected for analysis, since HEK293 cells exhibit a highly glycolytic metabolic phenotype—comparable to cancer cells—when grown under standard conditions, consuming glucose at a rate 7-fold greater than the next most utilized carbon source and converting most intracellular pyruvate into lactate (Henry *et al.*, 2011). The T-REx-293 cell line, a derivative of the HEK293 that stably expresses the tetracycline (Tet) repressor protein, was used as the basis for the experiments to allow for Tet-activated gene expression. Based on the T-REx-293 cell line, cells with Tet-inducible human *TRIB3* expression (TRIB3-293 cell line) and corresponding empty vector-transfected cells (Vector-293 cell line) were created (Örd *et al.*, 2007).

To determine if *TRIB3* expression is altered in response to glucose deficiency in HEK293 lineage cells, T-REx-293 cells were incubated in glucose-free growth medium and *TRIB3* mRNA expression was analyzed by RT-qPCR. The results show that following 3 h of glucose deprivation, *TRIB3* mRNA induction is already detectable, and subsequently the level of *TRIB3* expression increases further. The level of *TRIB3* unspliced precursor mRNA, which was quantified as a measure of gene transcription rate (Lipson and Baserga, 1989), is also increased during glucose deprivation, indicating that *TRIB3* is upregulated by transcriptional activation. In line with these data, Western blot analysis performed by D. Örd and presented in Ref. IV reveals that the level of TRIB3 protein is also strongly upregulated by glucose deficiency.

Next, several experiments were performed to elucidate the mechanisms leading to *TRIB3* induction in response to glucose deprivation. ATF4 is known to activate *TRIB3* gene transcription during ER stress and arsenite stress *via* a C/EBP–ATF composite site located in the *TRIB3* promoter region (Ohoka *et al.*, 2005; Örd and Örd, 2005). An analysis of ATF4 expression in T-REx-293 cells reveals that both the ATF4 protein and mRNA levels are upregulated during glucose starvation, raising the possibility that ATF4 also drives *TRIB3* gene activation in response to glucose deficiency. To test whether ATF4 mediates *TRIB3* activation in glucose-deprived cells, *ATF4* knockdown experiments were carried out. In cells suffering from glucose deprivation, *ATF4* silencing reduced the levels of *TRIB3* mRNA as well as *TRIB3* pre-mRNA, implicating ATF4 in the transcriptional activation of *TRIB3* during glucose deficiency.

Upregulation of the ATF4 protein level, a common response to diverse types of stress, is brought on by the phosphorylation of eIF2α (as described in section 2.3.3.2). Elucidating which particular kinase carries out eIF2α phosphorylation in glucose-deprived T-REx-293 cells would shed light on which cellular

functions are perturbed by the stress. One of the four eIF2α kinases, PERK, is characteristically activated in response ER stress (Wek *et al.*, 2006), a known possible consequence of glucose deficiency (El Mjiyad *et al.*, 2011). Overexpression of BiP (also known as GRP78 and HSPA5), a major ER chaperone protein, has been previously described to as a means to mitigate ER stress (Dorner *et al.*, 1992). In T-REx-293 cells, BiP overexpression blunted ATF4 protein induction in response to glucose withdrawal and also in cells treated with tunicamycin, a specific inducer of ER stress. Thus, ER stress is implicated in glucose deprivation-mediated ATF4 induction.

To verify that PERK activity initiates the signal for ATF4 induction, the highly selective PERK inhibitor GSK2606414 was employed (Axten *et al.*, 2012). The results show that PERK inhibition diminishes ATF4 protein induction in response to glucose deprivation or tunicamycin treatment but not in response to methionine deprivation, which is known to induce ATF4 by activating a different eIF2α kinase, GCN2 (Wek *et al.*, 2006). Concurrently, treatment with PERK inhibitor eliminates the upregulation of *TRIB3* mRNA and pre-mRNA in glucose-starved or tunicamycin-treated cells. Thus, TRIB3 is induced in glucose-deprived T-REx-293 cells as a transcriptional target of the ER stress response pathway consisting of the ER stress sensor kinase PERK and its transcriptional effector, ATF4.

4.9. Gene expression profiling of the pro-survival effect of TRIB3 in glucose-starved HEK293 cells (Ref. IV)

Insufficient availability of glucose disturbs multiple distinct facets of cell physiology. For instance, the ATP level declines, causing bioenergetic stress, the NADPH level decreases, impairing the antioxidant response and leading to oxidative stress, and a shortage of intermediates for protein glycosylation generates ER stress (El Mjiyad *et al.*, 2011). All of these manifestations of glucose starvation elicit responses from the cell and can conceivably contribute to cell death. Since TRIB3 has previously been found to affect the cellular responses to different types of stressful conditions (as discussed in section 2.4.1), *TRIB3* induction in glucose-deprived cells (noted above in section 4.8) could also signify an important regulatory role.

Cell viability analysis performed by D. Örd and included in Ref. IV revealed that Tet-induced *TRIB3* expression markedly increases TRIB3-293 cell survival during glucose starvation, while siRNA-mediated silencing of endogenous *TRIB3* in T-REx-293 cells promotes glucose deprivation-induced cell death. In Vector-293 cells, Tet treatment did not alter cell survival (D. Örd, Ref. IV) and had no effect of the level of *TRIB3* mRNA expression, indicating that Tet does not affect cell viability or *TRIB3* expression by unspecific mechanisms. These results demonstrate that TRIB3 enhances the viability of HEK293-derived cells suffering from glucose deficiency.

To gain insight into the effects of glucose deprivation on HEK293 lineage cells and how TRIB3 promotes glucose-deprived cell survival, genome-wide gene expression profiling was performed in TRIB3-293 and Vector-293 cells that had been either treated with Tet or left Tet-untreated and either incubated in complete medium or glucose-free medium. Notably, TRIB3 exhibits nuclear localization and interacts with several cellular stress-associated transcription factors (as discussed in section 2.4.1), giving rise to the possibility of transcriptional effects.

Remarkably, the results reveal that on a genome-wide scale, endogenous *TRIB3* mRNA ranks as the most highly glucose deprivation-regulated transcript. Overall, approximately 300 genes are upregulated and nearly 270 are downregulated by at least 2-fold in response to glucose starvation in HEK293-derived cells. These gene sets were analyzed with bioinformatical tools in order to gain insight into the state of the cells. Gene Ontology term enrichment analysis (Reimand *et al.*, 2011) was conducted for functional profiling of the genes, and transcription factor binding motif enrichment analysis (Janky *et al.*, 2014) was used to uncover possible regulatory mechanisms.

Among glucose deprivation-upregulated genes, the most prominent enrichment is revealed for ER stress response genes, in line with the notion of ER stress as a possible consequence of glucose deficiency (El Mjiyad *et al.*, 2011) and the root of glucose deprivation-induced *TRIB3* upregulation (4.8). Amino acid metabolism genes are also markedly enriched among glucose starvation-induced genes, possibly as a result of ER stress-induced ATF4 activating the amino acid availability program that it is known to control (Schroder and Kaufman, 2005; Siu *et al.*, 2002). The most highly enriched transcription factor binding motif in genes upregulated by glucose deprivation is the motif shared by ATF and C/EBP transcription factors, implicating these factors as major direct activators of the transcriptional response to glucose starvation.

On the other hand, glucose deprivation-downregulated genes are enriched for genes associated with cell cycle progression and display an over-representation of E2F transcription factor binding motifs, consistent with the cessation of cell proliferation in growth medium lacking glucose and the participation of E2F family members in the control of cell cycle progression (Duronio and Xiong, 2013).

To assess how elevated *TRIB3* expression affects the transcriptional response to glucose deprivation, all genes regulated by glucose starvation were filtered for genes demonstrating at least a 1.5-fold expression change in response to Tet-induced *TRIB3* expression. According to these criteria, *TRIB3* increases the expression of around 20 glucose deprivation-regulated genes, and a similar number of genes display an expression decrease. Among the glucose starvation-regulated genes that are also TRIB3-regulated, the direction of the TRIB3 effect tends to be towards the alleviation of the glucose deprivation-induced gene expression change. Notably, less than 10% of all glucose deprivation-regulated

genes are affected by TRIB3, indicating that TRIB3 is not a general regulator of the transcriptional response to glucose starvation but rather may perform a more targeted role.

Among the TRIB3-downregulated genes, functional profiling reveals an enrichment of amino acid metabolism-associated genes and transcription factor binding motif analysis predicts that the majority of the TRIB3-downregulated genes are target genes of ATF4. As discussed previously, TRIB3 is an inhibitor of ATF4 (2.4.1.1), which serves as the master activator of amino acid supply genes (Shan *et al.*, 2009). Thus, the results indicate that the predominant mechanism of TRIB3-mediated gene repression is the inhibition of ATF4. Curiously, ATF4 activity induces cell death during ER stress by increasing overall protein synthesis (Han *et al.*, 2013). However, the determination of the overall protein synthesis rate in TRIB3-293 cells by ^{35}S-methionine/cysteine pulse-labeling suggests that elevated *TRIB3* expression does not suppress cell death by an effect on overall protein synthesis.

Aside from the amino acid metabolism genes, the candidate TRIB3-regulated genes belong to diverse functional categories, including a number of genes that participate in other aspects of metabolism and several genes involved in cell communication. The gene that demonstrates the greatest expression sensitivity to TRIB3 is the TGF-β superfamily member inhibin βE (*INHBE*), which is expressed at a nearly 3-fold lower level in the presence of Tet-induced *TRIB3* in glucose-deprived TRIB3-293 cells. In comparison, the greatest TRIB3-mediated upregulation is seen for insulin-like growth factor-binding protein 2 (*IGFBP2*) mRNA, which is expressed at a level approximately 2.4-fold higher in glucose-deprived TRIB3-293 cells treated with Tet than in cells without Tet. Additionally, it is noteworthy that TRIB3-sensitive genes did not include classical cell death regulator genes, such as caspases or BCL2 family members.

4.10. Elevated *TRIB3* expression alleviates the glucose deprivation-induced downregulation of *IGFBP2*, a novel glucose deficiency survival factor (Ref. IV)

One of the genes that demonstrated relatively substantial TRIB3-dependence in the expression profiling experiment (described in the previous section) was *IGFBP2*. Humans have a total of six genes that encode IGFBPs, which bind the growth factors IGF-I and IGF-II in plasma, decreasing IGF availability but increasing its half-life (Baxter, 2014). Through this mode of action, IGFBPs can affect IGF receptor-dependent events such as IGF-induced cell proliferation (Hoflich *et al.*, 1998). However, IGFBPs also serve a range of IGF-independent roles, which may be intracellular as well as extracellular (Baxter, 2014). In particular, the IGF-independent actions of IGFBP2 include suppressing apoptosis by reducing caspase-3 activity (Migita *et al.*, 2010) and potentiating cell motility by activating integrin signaling (Schutt *et al.*, 2004). Clinically,

IGFBP2 is overexpressed in several types of human tumors, such as colorectal, breast, gastric, esophageal, pancreatic and ovarian cancer, glioma and leukemia (Busund *et al.*, 2005; Chen *et al.*, 2006; Fuller *et al.*, 1999; Mishra *et al.*, 1998; Wang *et al.*, 2006; Warnecke-Eberz *et al.*, 2015; Wex *et al.*, 1998; Zhang *et al.*, 2007). Further, high *in situ* IGFBP2 levels in leukemia and glioma are linked to poor prognosis (Dawczynski *et al.*, 2008; McDonald *et al.*, 2007), and high plasma IGFBP2 concentration is associated with poor prognosis in colorectal cancer and glioma patients (Lin *et al.*, 2009; Liou *et al.*, 2010). Therefore, IGFBP2 appears to be an interesting candidate for closer study in the context of the glucose deprivation stress response.

Following up on the results of the gene expression microarray, *IGFBP2* expression levels during glucose deprivation were examined further. RT-qPCR results demonstrate that in T-REx-293 cells incubated in glucose-free medium, *IGFBP2* mRNA expression is unaltered after 3 h of incubation but is measurably decreased after 6 h, eventually declining to 5-fold below the complete medium level after 48 h. Similarly, in Vector-293 and TRIB3-293 cells, *IGFBP2* mRNA expression declines in response to glucose deprivation. Crucially, however, in TRIB3-293 cells suffering from glucose deficiency, the level of *IGFBP2* mRNA is approximately 2-fold higher in Tet-treated cells compared to cells without Tet, verifying the microarray results. No effect of Tet on *IGFBP2* expression is seen in Vector-293 cells, irrespective of glucose availability, or in complete medium-incubated TRIB3-293 cells. These effects are also present at the protein level, with Tet treatment resulting in enhanced IGFBP2 protein levels in TRIB3-293 cells—but not in Vector-293 cells—during glucose deprivation. Thus, elevated *TRIB3* expression alleviates the glucose starvation-induced downregulation of the *IGFBP2* transcript and protein levels in HEK293 lineage cells.

Other researchers have demonstrated that *IGFBP2* expression inhibits cell death caused by genotoxic compounds (Migita *et al.*, 2010; Myers *et al.*, 2015; Zhu *et al.*, 2015). To discover whether *IGFBP2* expression can also regulate cell viability during glucose starvation, *IGFBP2* silencing and overexpression experiments were performed in T-REx-293 cells. Transfection of the cells with siRNA targeting *IGFBP2* resulted in the successful knockdown of *IGFBP2* expression, accompanied by an accelerated rate of cell death in response to glucose deprivation. Conversely, transfection of T-REx-293 cells with a human *IGFBP2* expression plasmid enhanced cell viability in glucose-free medium. These results demonstrate that reduced expression of *IGFBP2* aggravates cell death during glucose deprivation.

Potentiating *IGFBP2* expression could act as the mechanism by which TRIB3 defers glucose deprivation-induced cell death. To determine whether the pro-survival effect of TRIB3 during glucose starvation is mediated by IGFBP2, experiments incorporating the concurrent manipulation of *TRIB3* and *IGFBP2* expression were carried out. Using TRIB3-293 cells, the effect of *IGFBP2* silencing on glucose-deprived cell viability was analyzed with and without Tet-induced *TRIB3* expression. This revealed that in cells with diminished *IGFBP2*

expression, increased expression of *TRIB3* is unable to enhance survival. Complementing the previous experiment, the effect of concurrent and individual knockdown of *TRIB3* and *IGFBP2* on cell survival was examined in T-REx-293 cells. In addition to determining the incidence of cell death, visual changes in cell morphology were also monitored during the course of glucose starvation. The results show that simultaneously silencing *TRIB3* and *IGFBP2* impairs cell viability to a similar degree as silencing either of the genes individually. In line with this, glucose deprivation-induced cell morphology deterioration, such as cell rounding and detachment from substrate, is markedly accelerated in cells with individual or concurrent *TRIB3* and *IGFBP2* knockdown, compared to cells transfected with negative control siRNA only. Taken together, the results indicate that the cell viability-enhancing effect of TRIB3 is dependent on IGFBP2.

Uncovering TRIB3 and IGFBP2 as positive modulators of glucose-deprived cell survival implicates them in the stress responses acting in ischemic tissues and poorly-vascularized regions of tumors, examples of nutrient-deficient microenvironments. For instance, colorectal, ovarian and breast tumors are known to overexpress both *TRIB3* and *IGFBP2* (Bowers *et al.*, 2003; Busund *et al.*, 2005; Mishra *et al.*, 1998; Miyoshi *et al.*, 2009; Wang *et al.*, 2006; Wennemers *et al.*, 2011a; Xu *et al.*, 2007). Based on the results obtained from glucose-starved cells, the high *TRIB3* levels in these tumors could contribute to the enhanced levels of *IGFBP2* expression observed, which then could contribute towards cancer cell survival.

How TRIB3 regulates *IGFBP2* transcript abundance is currently unclear. Interestingly, glucose deprivation-regulated genes that are TRIB3-upregulated contain an overrepresentation of putative BCL6 binding sites, and *IGFBP2* is one of the predicted BCL6 target genes. BCL6, which is a transcription repressor (Chang *et al.*, 1996), has been found to form a complex with TRIB3 (Miles *et al.*, 2005). The functional significance of the interaction between TRIB3 and BCL6 has not been studied; it could be hypothesized that TRIB3 binding to BCL6 acts to de-repress BCL6 target genes. How IGFBP2 asserts its cell viability-enhancing effect on glucose-starved cells also remains obscure at present. TRIB3 interacts with and prevents caspase-3 activation (Shimizu *et al.*, 2012), and IGFBP2 is also known to negatively regulate caspase-3 activity (Migita *et al.*, 2010), suggesting a possible link that could be explored further.

5. CONCLUSIONS

Based on a review of previous literature, *TRIB3* is a mammalian gene that is transcriptionally induced in response to a variety of cellular stresses, including nutrient deprivation, oxidative stress and endoplasmic reticulum stress. In addition to promoter regulation, the control of *TRIB3* gene expression is known to occur at the level of mRNA degradation, protein post-translational modification and protein degradation. Further, several variants of human *TRIB3* mRNA have been described.

The TRIB3 protein features a protein kinase domain with deviations in highly-conserved, catalytically important motifs. Therefore, TRIB3 is a 'pseudokinase' that appears to lack marked enzymatic capability. TRIB3 is known to function by binding to specific proteins and affecting their activity, with the most common mode of action seen for TRIB3 being the inhibition of its interaction partner's activity. For example, TRIB3 forms inhibitory protein–protein interactions with ATF4, the central transcriptional regulator of the eIF2α phosphorylation pathway (also termed the integrated stress response), and Akt kinase, which is involved in glucose metabolism and cell survival. Notably, since ATF4 itself is a major transcriptional activator of *TRIB3* in response to certain stresses, a negative feedback loop controlling ATF4 activity is formed in cell cultures subjected to stress.

In all, nearly 30 different proteins have been reported to form interactions of known biological significance with TRIB3, including several transcription factors, protein kinases and ubiquitin ligases. Through these and potentially other interactions, TRIB3 participates in multiple intracellular signaling pathways and is involved in the cellular stress response and the determination of cell viability, glucose signaling and energy metabolism, inflammation, and cell differentiation. As a result, TRIB3 is implicated in several human diseases, notably cancer and type 2 diabetes. Nevertheless, much regarding *TRIB3* function remains unclear. Drawing upon the gaps in the current knowledge, several investigations into the regulation and role of *TRIB3* were undertaken in the current work.

The following conclusions can be drawn from the results of the experiments:

- The *TRIB3* mRNA population composition changes in response to stress: in HepG2 human hepatoma cells treated with arsenite, an inducer of oxidative stress, *TRIB3* mRNA variants containing a truncated 5′-UTR become predominant, resulting in enhanced translational potential of the *TRIB3* mRNA pool.
- *Trib3* expression is positively regulated by the growth factor IL-3 in BMMCs, mast cells derived *in vitro* from mouse bone marrow, and BMMCs generated from *Trib3*-deficient mice display impaired immunological functions (both degranulation and cytokine induction), implicating *Trib3* in the modulation of the immune response.

- *Trib3* expression increases during the course of mouse brain development, and mice with a lack of *Trib3* exhibit enlarged lateral ventricles in the brain. In the adult mouse brain, *Trib3* expression is constitutively high in the cerebellum and inducible in the anterior piriform cortex by the consumption of amino acid-deficient diet. However, *Trib3* knockout mice display normal aversion to amino acid-imbalanced diet, as well as unaltered long-term spatial memory and fear memory, indicating that eIF2α–ATF4 pathway-dependent cognitive functions are not altered by the absence of *Trib3*.
- Endoplasmic reticulum stress-mediated ATF4 activation upregulates *TRIB3* transcription in glucose-deprived cells of the HEK293 lineage. Genome-wide gene expression profiling revealed that *TRIB3* mRNA ranks as the most highly glucose deprivation-regulated transcript after 24 h of glucose starvation, and identified approximately 40 glucose deprivation-responsive genes that are affected by *TRIB3* overexpression. Transcription factor motif enrichment analysis uncovered that most TRIB3-inhibited genes are probable target genes of ATF4, a known TRIB3 binding partner. Additionally, TRIB3 substantially alleviated the repression of *IGFBP2* in glucose-deprived cells, which was shown to be a novel mechanism of deferring cell death caused by glucose deficiency. Thus, TRIB3 and IGFBP2 are potentially important for cell survival in nutrient-poor microenvironments such as tumors or infarction core regions.

SUMMARY IN ESTONIAN

Imetajate pseudokinaasi TRIB3 funktsioonid ja regulatsioon

TRIB3 on üks kolmest imetajates esinevast homoloogist äädikakärbse valgule Tribbles, mis avastati kui mitoosi inhibiitor kärbse varajases embrüonaalses arengus. Embrüotes, milles puudus funktsionaalne Tribbles valk, nähti gastrulatsiooni faasis rakkude pidurdamatut paljunemist, mistõttu nimetati geen ulmeseriaali „Star Trek" tegelaste *tribble*'ite järgi, kellele on iseloomulik ülikiire arvukuse suurenemine.

Tribbles perekonna valgud sisaldavad ühte selgesti tuvastatavat valgudomeeni, milleks on seriini/treoniini proteiinikinaasi domeen. Kuna aga mitmed selle domeeni katalüütiliselt olulised ja laialdaselt konserveerunud aminohappejäägid on Tribbles valkudes asendunud, klassifitseeritakse Tribbles valgud pseudokinaasideks. Kokku on inimeses pseudokinaase ligikaudu kümme protsenti kõigist kinaasi domeeni sisaldavatest valkudest ning valgujärjestuste järgi paigutuvad pseudokinaasid paljudesse proteiinikinaaside alamklassidesse. Oluliste konserveerunud aminohappejääkide asendumine viitab sellele, et pseudokinaasid ei saa teostada kinaasi ensüümreaktsiooni klassikalise katalüütilise mehhanismi vahendusel. Samas ei pruugi pseudokinaasid olla ensümaatilises mõttes täiesti inertsed; muuhulgas on ka TRIB3 puhul hiljuti leitud (väga) vähene autofosforüleerimise võime *in vitro*.

Nii inimesel kui ka hiirel on *TRIB3* mRNA ekspressioonitase kõrge maksas ning paljudes teistes organites ja kudedes on avaldumise tase mõõdukas. Tähelepanuväärne on, et *TRIB3* ekspressioonitase tõuseb paljudes rakutüüpides vastusena mitmesugustele stressidele. Näiteks aktiveeritakse *TRIB3* ekspressioon endoplasmiitilise retiikulumi (ER) stressi, asendamatu aminohappe puuduse, hapnikupuuduse ja oksüdatiivse stressi korral (kuid mitte genotoksilise stressi korral). On leitud, et stresside korral vahendab *TRIB3* promooteri aktivatsiooni DNA element, mida nimetatakse C/EBP-ATF liitelemendiks, ning sinna seondub stressi korral transkriptsioonifaktor ATF4, moodustades heterodimeeri mõne C/EBP perekonna faktoriga (näiteks CHOP, C/EBPγ või C/EBPβ). Imetajarakkudes toimub ühtse vastusena eritüübilistele stressidele translatsiooni initsiatsioonifaktori eIF2α fosforüleerimine, mis toob kaasa ATF4 translatsioonilise ülesreguleerimise – seeläbi on seletatav *TRIB3* ekspressiooni käivitumine vastusena eritoimelistele stressoritele. Lisaks transkriptsiooni tasemele toimub *TRIB3* avaldumise reguleerimine veel ka mRNA stabiilsuse mõjutamise, posttranslatsiooniliste modifikatsioonide ja valgu eluea reguleerimise kaudu.

TRIB3 valk asetseb rakutuumas ja tsütoplasmas ning ta funktsioneerib teiste valkudega seondumise kaudu. TRIB3 enamasti inhibeerib oma seondumispartneri aktiivsust, kuid mõnel juhul mõjutab ka partneri spetsiifikat. Praeguseks on selliseid TRIB3 interaktsioonipartnereid, mille puhul on interaktsioonile leitud ka mingisugune bioloogiline tähtsus, teada ligi 30 tükki. On tähelepanuväärne, et TRIB3 inhibeerib mitmeid transkriptsioonifaktoreid, mis tema enda avaldumistaset suurendavad, moodustades seega negatiivse tagasiside mehha-

nisme (näiteks ATF4, CHOP, C/EBPβ ja NF-κB puhul). TRIB3 interakteerub veel ka mitmete kinaasidega (enim tähelepanu on pälvinud interaktsioon kinaasiga Akt), ubikvitiini ligaasidega ja muudesse klassidesse kuuluvate valkudega. Mitmed TRIB3 funktsioonid on seotud raku stressivastuse ja rakkude elulemuse reguleerimisega, näiteks stressivastuse transkriptsioonifaktorite ATF4, CHOP ja NF-κB inhibeerimine, rakkude elulemusega seotud kinaaside Akt ja MLK3 inhibeerimine ning kaspaas-3 aktiveerimise mõjutamine. Seejuures võib TRIB3 sõltuvalt stressi- ja rakutüübist avaldada elulemusele positiivset või negatiivset mõju.

Hiired, kellel puuduvad mõlemad koopiad *Trib3* geenist (*Trib3*$^{-/-}$ hiired), on eluvõimelised ja ei eristu väliselt metsiktüüpi (*Trib3*$^{+/+}$) hiirtest. Rasvarikka söödaga toitmise korral on aga leitud, et *Trib3*$^{-/-}$ hiirtel on paranenud glükoosi ainevahetuse näitajad. Teisest küljest on *Trib3* puudusega hiirtel suurenenud vastuvõtlikkus diabeedist tingitud neerupatoloogia tekkeks ja kiirenenud kasvajate areng mõnedes kasvajatekke mudelites. Lisaks on valmistatud ka transgeenseid hiiri, kellel on *Trib3* teatud koes üleekspresseeritud, ning nende hiirte uurimisel saadud tulemused viitavad, et TRIB3 võib mõjutada kõhunäärme β-rakkude, rasvkoe ja lihaskoe toimimist. Inimeses on TRIB3 avaldumistase kõrgenenud erinevates pahaloomulistes kasvajates ning jämesoole, maksa ja kopsu kasvajate puhul on kõrgenenud TRIB3 tase seotud halvema prognoosiga. Lisaks vähkkasvajatele on TRIB3 inimeses palju tähelepanu pälvinud seoses 2. tüüpi diabeediga ning sellega kaasnevate haigustega. Nimelt esineb inimpopulatsioonides *TRIB3* alleel, millelt kodeeritavas valgus on 84. positsioonis glutamiinhappe jääk asendunud arginiini jäägiga, ja on leitud, et selle alleeli esinemine toob kaasa 2. tüüpi diabeeti haigestumise riski suurenemise.

Varasemast on teada, et inimese *TRIB3* geenilt toodetakse erinevaid mRNA isovorme, millel varieerub 5′-mittetransleeritav regioon, sest kasutatakse alternatiivseid variante esimesest eksonist ja alternatiivseid transkriptsiooni alguspunkte. Käesoleva töö üheks eesmärgiks oli kirjeldada, milline on *TRIB3* mRNA-de populatsiooni isovormiline koosseis ja uurida, kas see muutub rakustressi tingimustes. Selle saavutamiseks töötati välja reaalaja PCR-il põhinev uuringusüsteem, mis võimaldab omavahel võrrelda erinevate mRNA variantide arvukust. Süsteemi rakendati inimese hepatoomi rakuliini HepG2 rakkudes, mida inkubeeriti eelnevalt kas tavatingimustel või arseniiti sisaldavas söötmes, mis põhjustab rakkudes oksüdatiivset stressi ja suurendab *TRIB3* mRNA avaldumistaset.

Tulemustest selgub, et nii tavasöötmes kui ka arseniidistressi korral domineerib splaiss-variant 1A, moodustades ligikaudu 80% *TRIB3* mRNA-dest, arvukuselt järgmine on 1B4 (ligikaudu 8%) ning variandid 1B1, 1B2 ja 1B3 on minoorsed (kokku ligikaudu 1%). Tähelepanuväärne on, et splaiss-variant 1A mRNA-de hulgas toimub vastusena stressile oluline muutus transkriptsiooni alguspunktide kasutamises: kui tavatingimustel kasvatatud rakkudes oli vaid ligikaudu 10% 1A mRNA-dest lühikese, C/EBP-ATF liitelementidest allavoolu algava 5′-liiderjärjestusega, siis arseniidiga töödeldud rakkudes oli selliste mRNA-de osakaal ligikaudu 80% splaiss-variant 1A mRNA-dest. Seega toimub

arseniidistressi korral *TRIB3* mRNA avaldumistaseme tõus peamiselt lühikest 5′-liiderjärjestust sisaldavate splaiss-variant 1A mRNA-de tootmise kaudu. Meie laboris D. Ördi poolt teostatud katsed näitavad, et valgu tootmine taolistelt mRNA-delt on efektiivsem ja seega toimivad stressi korral *TRIB3* geeniekspressiooni suurendamiseks nii transkriptsioonilised kui ka translatsioonilised mehhanismid.

Nuumrakud on immuunrakud, mis paiknevad kudedes (näiteks nahas ja limaskestades) ja viivad ellu IgE-vahendatud immuunvastuseid, muuhulgas ka allergilisi reaktsioone. Antigeeniga kokkupuutel eritavad aktiveeritud nuumrakud põletikumediaatoreid, näiteks histamiini, prostaglandiine ja tsütokiine (sh TNF ja IL-6). Imetajates on Tribbles valkudel varasemalt leitud mõningaid seoseid vereloome protsessidega, näiteks TRIB3 puhul vere punaliblede moodustumisega ning TRIB1 ja TRIB2 puhul leukeemia tekkega, kuid nuumrakkude osas andmed puuduvad.

Nuumrakkude eellased paiknevad luuüdis ning *in vitro* on nuumrakke võimalik hiire luuüdist toota, kasvatades luuüdi rakke kasvufaktori IL-3 juuresolekul. Uurides taoliselt saadud primaarseid nuumrakukultuure, leiti käesolevas töös, et *Trib3* on transkriptsioonilisel tasemel positiivselt reguleeritud nuumrakkude kasvufaktori IL-3 poolt. Võrdluseks, nuumrakkude eksponeerimine IgE antikehale ja stimuleerimine antigeeniga avaldasid *Trib3* ekspressioonitasemele palju nõrgemat mõju. Järgnevalt loodi nuumrakukultuurid, milles puudus *Trib3* geen, kasutades meie töögrupi poolt loodud *Trib3* deletsiooniga hiireliini. Selgus, et *Trib3* puudumine ei häiri nuumrakkude diferentseerumist IL-3 toimel *in vitro*, kuid *Trib3*$^{-/-}$ nuumrakkude immunoloogilises aktiivsuses avaldusid mitmed kõrvalekalded. Nimelt on antigeeniga stimuleeritud *Trib3*$^{-/-}$ nuumrakkudes oluliselt pärsitud nii põletikumediaatorite vabastamine graanulitest (degranulatsioon) kui ka tsütokiinide *de novo* sünteesi käivitamine, mis kujutavad endast kaht võrdlemisi eraldi toimuvat immunoloogilise aktiivsuse faasi. Seega võib *Trib3* osaleda immuunvastuste mõjutamises. *In vivo* katse uurimaks nuumrakkude degranulatsiooni hiire kõrva nahas ei näidanud erinevust *Trib3*$^{+/+}$ ja *Trib3*$^{-/-}$ hiirte vahel, kuid kuna IL-3 mõjul luuüdist *in vitro* moodustunud nuumrakud meenutavad pigem limakestades paiknevaid nuumrakke kui naha nuumrakke, siis organismi tasemel võib *Trib3* puudumise mõju avalduda näiteks limaskestade nuumrakkudes või teistes nuumrakkude füsioloogilistes reaktsioonides peale degranulatsiooni, mille uurimisele teostatud katse peamiselt suunatud oli.

Trib3 mRNA ja selle poolt kodeeritav valk avastati esmalt neuronaalsetes rakkudes ning ATF4 – TRIB3 poolt inhibeeritav stressivastuse transkriptsioonifaktor – on kirjeldatud lisaks ka kui pikaajalise mälu vaigistaja. Seetõttu seati käesoleva töö üheks eesmärgiks selgitada *Trib3* rolli peaajus. Esmalt uuriti *Trib3* mRNA avaldumistaset hiire peaaju embrüonaalse arengu vältel ja leiti, et *Trib3* ekspressioon ajus suureneb sujuvalt embrüonaalse arengu 14. päevast (E14) kuni 18. päevani (laborihiirte tiinus kestab 19–20 päeva). Vahetult sündimise järel langeb *Trib3* ekspressioonitase mõnevõrra, kuid taastub kolmandaks sündimisjärgseks päevaks, ületades E14 taset

selleks ajaks ligikaudu kuus korda. Seevastu *Trib1* ja *Trib2* avaldumistasemed on peaaju arengu vältel suhteliselt stabiilsed ja ei ole muutunud ka juveniilsete *Trib3*$^{-/-}$ hiirte ajus.

Morfoloogilised uuringud näitasid, et *Trib3* puudusega hiirtel on peaaju ehitus üldjoontes normaalne, kuid avaldus trend, et *Trib3*$^{-/-}$ hiirtel on suuremad külgmised ajuvatsakesed. Täiendavad mõõtmised kinnitasid, et nii täiskasvanud kui ka juveniilsetel *Trib3*$^{-/-}$ hiirtel on külgmised ajuvatsakesed poolteist kuni kaks korda suuremad kui *Trib3*$^{+/+}$ hiirtel. Seega võib *Trib3* olla seotud ajuvatsakeste arenguga. Inimese puhul on leitud, et ajuvatsakesed suurenevad vananemise ja neurodegeneratiivsete haiguste korral, kuid samas on ajuvatsakeste mõõtmed ka tervetel noortel inimestel suuresti varieeruvad.

Kuna TRIB3 on rakustressi kontekstis kirjeldatud kui eIF2α-ATF4 raja tagasisideline inhibiitor ning seesama rada on seotud ka mitmete käitumuslike protsessidega, siis otsustati läbi viia *Trib3* ekspressiooni uuringuid hiire peaaju erinevates osades ja teostada käitumiskatseid *Trib3*$^{-/-}$ hiirtega. Hiired on võimelised tunnetama teatud ajuregiooni, *anterior piriform cortex*-i (APC) abil asendamatute aminohapete ebatasakaalulist esinemist äsjatarbitud söödas ja piiravad sellise sööda tarbimist. Reaalaja PCR-i abil saadud tulemused näitavad, et tavatingimustel on *Trib3* mRNA avaldumistase ligikaudu ühesugune suuraju frontaalkorteksis, hipokampuses ja APC-s ning märkimisväärselt kõrgem väikeajus (seevastu *Trib1* ja *Trib2* tasemed varieerusid uuritud ajuosade võrdluses vähe). Leutsiinivaba toidu tarbimise järel suurenes aga *Trib3* ekspressioon spetsiifiliselt APC-s. Hiirte toidutarbimise jälgimine näitas, et *Trib3*$^{-/-}$ hiirte vastumeelsus leutsiinivaba sööda tarbimisele on võrreldav *Trib3*$^{+/+}$ hiirte omaga. Lisaks toidu aminohappelise koostise tunnetamisele osaleb eIF2α-ATF4 rada ajus veel pikaajalise ruumimälu ja hirmumälu toimimises. Vastavad käitumiskatsed aga näitasid, et *Trib3*$^{-/-}$ hiirtel ei ole muutunud ruumimälu, ruumimälu ümberõppimise võime ega auditoorne või kontekstuaalne hirmumälu. Seega ei ole TRIB3 valgul tõenäoliselt asendamatut rolli eIF2α-ATF4 raja aktiivsuse piiramisel peaajus käitumuslike protsesside raames, kuid *Trib3* eripärane ekspressioonimuster ajus ja ajuvatsakeste suuruse fenotüüp *Trib3*$^{-/-}$ hiirtel viitavad, et TRIB3 võib täita ajus mõnda muud rolli.

Glükoosipuudus, mis esineb näiteks tahkete kasvajate sisemuses ja infarktikolletes, põhjustab rakkudes stressi ning võib kutsuda esile rakusurma. Käesolevas töös uuriti TRIB3 olulisust glükoosipuuduse stressivastusele, kasutades HEK293 rakkudest stabiilse transfektsiooni teel valmistatud rakuliine, mis võimaldavad rakendada tetratsükliini poolt aktiveeritavat geenieksprессiooni süsteemi.

Reaalaja PCR-i abil saadud tulemused näitavad, et glükoosipuuduse korral tõuseb rakkudes *TRIB3* mRNA avaldumistase kiiresti (alates 3 h) ja et *TRIB3* mRNA ülesregulatsioon tuleneb *TRIB3* geeni transkriptsioonilisest aktivatsioonist. *ATF4* vaigistamise katsed näitasid, et ATF4 on vajalik *TRIB3* ülesregulatsiooniks glükoosipuuduse korral. Täiendavad uuringud selgitasid välja, et ATF4 aktivatsioon (ja *TRIB3* geeni ülesregulatsioon) glükoosipuuduse korral toimub ER stressi tõttu ja on vahendatud ER-seoselise eIF2α kinaasi PERK poolt.

D. Ördi poolt läbiviidud katsete põhjal toetab *TRIB3* glükoosipuuduse tingimustes rakkude elulemust. Selgitamaks *TRIB3* mõju glükoosipuuduse transkriptsioonilisele vastusele teostati ülegenoomne geenieksprессiooni analüüs. Glükoosipuuduse toimel oli muutunud sadade geenide ekspressioonitase, kusjuures ülesreguleeritud geenide hulgas oli kõige tugevamalt üleesindatud geenide kategooriaks ER stressi vastuse geenid ja kõige tugevamalt üleesindatud transkriptsioonifaktori seondumismotiiviks oli C/EBP ja ATF transkriptsioonifaktorite ühendmotiiv. See viitab, et C/EBP ja ATF perekondade faktorid on olulised otsesed transkriptsioonilised aktivaatorid glükoosipuuduse vastuse korral. Selleks, et selgitada TRIB3 toimet glükoosipuuduse vastusele, vaadeldi *TRIB3* üleekspressiooni mõju glükoosipuuduse poolt mõjutatud geenidele. Kokku tuvastati ligikaudu 40 TRIB3-tundlikku geeni. Analüüsides transkriptsioonifaktorite seondumismotiivide esinemist TRIB3 toimel allareguleeritud geenides, selgus, et ATF4 inhibeerimine võib olla peamine mehhanism, mille kaudu TRIB3 geenieksprессiooni represseerib. Seevastu geenidel, mille ekspressioonitase suurenes TRIB3 toimel, ei avaldunud nii selget ühendavat mehhanismi.

Glükoosipuuduse vastuse geen, mille ekspressioon TRIB3 toimel enim tõusis, oli *IGFBP2*. Täiendavad katsed kinnitasid, et *IGFBP2* mRNA ja valgu tasemed langevad glükoosipuuduse korral ning et TRIB3 taseme tõstmine leevendab märkimisväärselt IGFBP2 taseme vähenemist glükoosipuuduse korral. *IGFBP2* on kliinilises mõttes tähelepanuväärne geen, kuna selle üleekspressiooni on kirjeldatud paljudes inimese kasvajates ja mõnedes kasvajatüüpides on *IGFBP2* suurem avaldumistase ka kehvema prognoosi markeriks. Samas on võrdlemisi vähe teada *IGFBP2* mõjust rakkude elulemusele. *IGFBP2* vaigistamise ja üleekspressiooni katsete abil õnnestus välja selgitada, et *IGFBP2* avaldumistaseme langus glükoosipuuduse korral toetab rakkude suremist, seega võib IGFBP2 hulga tõstmine olla mehhanism, mille kaudu TRIB3 rakkude elulemust parandab. Järgnevad katsed, kus mõjutati üheaegselt *IGFBP2* ja *TRIB3* ekspressioonitasemeid, näitasid, et *TRIB3* positiivne mõju rakkude elulemusele on tõepoolest sõltuv *IGFBP2*-st. Seega on *TRIB3* ja *IGFBP2* glükoosipuuduse all kannatavate rakkude elulemuse positiivsed mõjutajad, mis võivad mängida tähtsat rolli näiteks käärsoole, munasarja ja rinna vähkkasvajates, kus on kirjeldatud nii *TRIB3* kui ka *IGFBP2* üleekspressioon.

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CURRICULUM VITAE

Name: Tiit Örd
Date of birth: December 5, 1986
Citizenship: Estonian
Address: Estonian Biocentre, Riia 23b, 51010 Tartu, Estonia
Telephone: +372 737 5044
E-mail: tiit.ord@gmail.com

Education:

2010–…	University of Tartu, Gene Technology, PhD student
2008–2010	University of Tartu, Gene Technology, MSc
2005–2008	University of Tartu, Gene Technology, BSc, *cum laude*
2002–2005	Hugo Treffner Gymnasium, Department of Life Sciences

Professional employment:

2014–2015	Estonian Biocentre, junior researcher

Main research interests:
Stress responses in mammalian cells, regulation of gene expression, animal models, functional genomics

Supervised theses:
Tuuliki Biene, master's thesis, 2015 (co-supervisor: Dr. Tõnis Örd)
Martin Kala, bachelor's thesis, 2014 (co-supervisor: Prof. Jaanus Remme)
Tuuliki Biene, bachelor's thesis, 2013 (co-supervisor: Dr. Sulev Ingerpuu)

List of publications:

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ELULOOKIRJELDUS

Nimi: Tiit Örd
Sünniaeg: 5. detsember 1986
Kodakondsus: Eesti
Aadress: Eesti Biokeskus, Riia 23b, 51010 Tartu, Eesti
Telefon: +372 737 5044
E-post: tiit.ord@gmail.com

Haridustee:

2010–…	Tartu Ülikool, geenitehnoloogia, doktorant
2008–2010	Tartu Ülikool, geenitehnoloogia, MSc
2005–2008	Tartu Ülikool, geenitehnoloogia, BSc, *cum laude*
2002–2005	Hugo Treffneri Gümnaasium, loodussuund

Töökogemus:

2014–2015	Eesti Biokeskus, nooremteadur

Peamised teaduslikud huvid:
Stressivastused imetajarakkudes, geeniekspressiooni mehhanismid, loom-mudelid, funktsionaalne genoomika

Juhendatud lõputööd:
Tuuliki Biene, magistritöö, 2015 (kaasjuhendaja: Dr. Tõnis Örd)
Martin Kala, bakalaureusetöö, 2014 (kaasjuhendaja: Prof. Jaanus Remme)
Tuuliki Biene, bakalaureusetöö, 2013 (kaasjuhendaja: Dr. Sulev Ingerpuu)

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