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TRIB3 limits FGF21 induction during *in vitro* and *in vivo* nutrient deficiencies by inhibiting C/EBP–ATF response elements in the *Fgf21* promoter

Tiit Örd, Daima Örd, Tõnis Örd*

Estonian Biocentre, Institute of Genomics, University of Tartu, Riia 23b, 51010 Tartu, Estonia

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ABSTRACT

Mammals must be able to endure periods of limited food availability, and the liver plays a central role in the adaptation to nutritional stresses. TRIB3 (Tribbles homolog 3) is a cellular stress-inducible gene with a liver-centric expression pattern and it has been implicated in stress response regulation and metabolic control. In the current article, we study the involvement of TRIB3 in responses to nutrient deficiencies, including fasting for up to 48 h in mice. We show that hepatic expression of *Trib3* is increased after 48 h of fasting and mice with a targeted deletion of the *Trib3* gene present elevated hepatic triglyceride content and liver weight at 48 h, along with an upregulation of lipid utilization genes in the liver. Further, hepatic and serum levels of the metabolic stress hormone FGF21 are considerably increased in 48-h-fasted *Trib3* knockout mice compared to wild type. *Trib3* deficiency also leads to elevated FGF21 levels in the mouse liver during essential amino acid deficiency and in cultured mouse embryonic fibroblasts during glucose starvation. Reporter assays reveal that TRIB3 regulates FGF21 by inhibiting ATF4-mediated, C/EBP–ATF site-dependent activation of *Fgf21* transcription. Based on chromatin immunoprecipitation from mouse liver, the binding of TRIB3 and ATF4, a transcription factor known to physically interact with TRIB3, is significantly increased at the *Fgf21* promoter following 48 h of fasting. Thus, under nutrient-limiting conditions that stimulate ATF4 activity, TRIB3 is implicated in the regulation of metabolic adaptation by restraining the transcription of *Fgf21*.

1. Introduction

TRIB3 (also known as TRB3, NIPK, SKIP3 and SINK) is a pseudokinase, *i.e.*, it contains a protein kinase domain with non-consensus catalytic motifs and an apparent lack of biologically significant kinase activity, and, as such, TRIB3 asserts its known biological effects by modulating the activity of other proteins through protein–protein interactions (reviewed in [1,2]). TRIB3 is reported to form inhibitory interactions with transcription factors that mediate cellular stress responses (ATF4 and CHOP) and inflammation (NF-κB) and kinases involved in metabolic control (Akt, mTORC2 and AMPK) [1,2]. A characteristic feature of TRIB3 expression is its inducibility in response to diverse cellular stress conditions, such as endoplasmic reticulum (ER) stress, oxidative stress, glucose deficiency, and insufficient amino acid supply [3–8]. Notably, the TRIB3-inhibited proteins ATF4 and CHOP directly activate *TRIB3* expression, providing an opportunity for negative feedback control of stress response transcription [3,7,9,10].

The metabolic functions of TRIB3 at the organism level remain incompletely understood. *Trib3*-deficient mice have been generated by several groups (summarized in [1]), and under standard conditions *Trib3* knockout mice present normal body weight, body composition, metabolic rate and glucose homeostasis [11–14]. However, in circumstances that promote hyperglycemia (such as high-fat diet feeding), the loss of *Trib3* appears to alleviate insulin resistance [15,16]. In a basal (fed) physiological state, TRIB3 displays a liver-centric expression pattern in both mice and humans [4,17].

During the course of their life, many mammals must endure periods of limited food availability. The body copes with fasting by mobilizing its energy reserves in a concerted multi-organ response [18,19]. The liver plays a central role in the metabolic adaptation to fasting, as it is a major site responsible for the supply of carbohydrates (glycogen hydrolysis and gluconeogenesis), for the processing of adipose-derived non-estrified fatty acids into a readily utilized energy substrates (ketogenesis), and for the catabolism of amino acids derived from skeletal muscle protein breakdown [19–21]. The reprogramming of liver metabolism in response to fasting involves the coordinated modulation of transcriptional 'metabolism master regulators', for example the activation of PPARα and the suppression of SREBP1c and ChREBP [18–21].

Abbreviations: ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; ER, endoplasmic reticulum; FGF21, fibroblast growth factor 21; MEF, mouse embryonic fibroblast; PPARα, peroxisome proliferator-activated receptor α; TRIB3, Tribbles homolog 3

* Corresponding author.
E-mail address: tord@ebc.ee (T. Örd).

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Possibly as a response to the demanding metabolic functions, during fasting the liver displays an activation of defenses against ER stress and oxidative stress [18,20].

An important liver-derived regulator during starvation is the metabolic stress hormone fibroblast growth factor 21 (FGF21), which has attracted much attention for its effects on glucose and lipid metabolism [22–24]. FGF21 is an endocrine member of the fibroblast growth factor family, acting as a systemic metabolic regulator [23] that is secreted into the bloodstream by the liver during fasting [25] and upon the consumption of amino acid-deficient diet [26]. The targets for circulating FGF21 are believed to include adipose tissue, the central nervous system, and, *via* paracrine action, the liver itself [23]. In the current article, we study how adult *Trib3* knockout mice respond to a period of starvation up to 48 h in duration, in comparison to wild type mice. We find that hepatic and serum FGF21 levels are markedly elevated in 48 h-starved *Trib3*-deficient mice, and, while *Trib3*-deficient individuals maintain normal blood glucose, glucose tolerance and rate of body mass loss, they present a prolonged accumulation of triglycerides in the liver. Subsequent analyses uncovered that *Trib3* functions as a negative regulator of *Fgf21* transcription in multiple types of nutrient deficiency conditions.

2. Results

2.1. Hepatic expression of Trib3 increases during extended fasting and the livers of Trib3$^{-/-}$ mice display a prolonged accumulation of triglycerides upon fasting

Since *Trib3* affects cell responses to multiple types of nutritional stresses *in vitro* [3,7], we wondered whether a lack of *Trib3* leads to an altered response to fasting *in vivo*. Previous results, obtained under standard dietary conditions, have revealed that a major site of *Trib3* expression in the body is the liver [4,13], an organ that is central in the metabolic adaptation to starvation conditions [19,21]. To analyze the effect of fasting on *Trib3* expression, wild type mice were deprived of food for up to 48 h, followed by tissue collection. The results show that fasting for 48 h leads to a substantial increase in the level of *Trib3* expression in the liver, compared to 0 h (Fig. 1A). Further, in a state of prolonged (36–48 h) fasting, the level of *Trib3* expression in the liver is considerably higher than that in white adipose tissue or skeletal muscle (Fig. 1B), organs which also carry out specific roles in fasting physiology.

As shown in Fig. 1C, the body weight of *Trib3*-deficient mice fed a chow diet *ad libitum* is unaltered compared to wild type mice for both males and females, in agreement with previous reports [13,14]. Moreover, there is also no significant difference in the body weights of *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice after 48 h of fasting, which leads to an approximately 25% loss of body weight in both males and females (Fig. 1C). There is also no change in the time course of body weight loss during fasting (0–36 h) in *Trib3* knockout mice (Supplementary Fig. S1A). TRIB3 has been reported to promote insulin resistance in high-fat diet-fed mice [15,16], and prolonged fasting necessitates a reorganization of carbohydrate metabolism to apportion glucose usage between organs [19,21]. Therefore, we also characterized glucose homeostasis in fasted *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice. As expected, blood glucose levels decrease as fasting progresses from 24 to 48 h; however, no significant difference is evident between *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice (Fig. 1D). A glucose tolerance test performed after 24 h of fasting also reveals similar results for both genotypes (Supplementary Fig. S1B). Thus, *Trib3*-deficient mice subjected to prolonged food deprivation display grossly normal body weight loss and glucose homeostasis.

In one of the major metabolic shifts occurring upon fasting, large amounts of fatty acids are released by white adipose tissue and imported into the liver for utilization as an energy source [19]. Since this fatty acid influx initially exceeds the hepatic capacity for β-oxidation, fatty acids are temporarily stored in hepatocytes in the form of triglycerides, manifesting in transient hepatic steatosis [19]. As shown in Fig. 1E, 24 h of starvation leads to an approximately 5-fold increase in hepatic triglyceride content in wild type mice, and a similar response is seen in *Trib3*$^{-/-}$ mice. However, after 48 h of fasting, liver triglyceride levels in wild type mice have decreased, while in *Trib3*-deficient mice, liver triglyceride levels remain essentially unchanged compared to their 24 h level (Fig. 1E). In comparison, skeletal muscle triglycerides, which are also utilized during fasting [27], are present at similar amounts in *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice starved for 48 h (Supplementary Fig. S1C). In line with increased hepatic triglyceride accumulation, liver weight is elevated in *Trib3*-deficient mice, compared to wild type mice, after 48 h of fasting (Fig. 1F). Thus, a lack of *Trib3* manifests in a prolonged period of hepatic steatosis upon starvation.

2.2. Gene expression analysis of key metabolic pathways in the liver and skeletal muscle of fasted Trib3$^{-/-}$ mice

In the liver, as well as other organs, metabolic adaptation to fasting is achieved through extensive transcriptional regulation involving a number of transcription factors [18,20]. To gain insight into the delayed hepatic triglyceride clearance in fasted *Trib3*-deficient mice, RT-qPCR was used to analyze sets of genes involved in starvation responses in the liver and in skeletal muscle.

During fasting, lipid metabolism in the liver is rearranged to carry out the breakdown of adipose-derived fatty acids for use as an energy source for the body, and central to this is an upregulation of hepatic fatty acid β-oxidation [19,20]. When *Trib3*$^{-/-}$ mice are fed *ad libitum* or starved for 24 h, their hepatic expression levels of the β-oxidation genes CPT2, ACOX1 and CPT1a are similar to wild type mice (Fig. 2A). However, after 48 h of fasting, *Trib3*$^{-/-}$ mouse livers display enhanced mRNA levels for CPT2, an enzyme crucial for mitochondrial β-oxidation, and ACOX1, the initial enzyme for peroxisomal β-oxidation, compared to *Trib3*$^{+/+}$ mice (Fig. 2A). There is also a trend towards elevated hepatic mRNA expression of PGC1α, a master regulator of mitochondrial respiratory capacity, in 24- and 48-h-starved *Trib3*$^{-/-}$ mice compared to wild type mice ($p = 0.07$ and 0.09 for 24 and 48 h, respectively; Fig. 2A). The expression levels of CPT1a, the partner enzyme for CPT2 in shuttling fatty acids into the mitochondrial matrix, remain unaffected by *Trib3* deletion during fasting, as are the mRNA levels of PPARα, a transcription factor that regulates many lipid utilization genes in a ligand-dependent manner (Fig. 2A).

To handle the increased adipose tissue lipolysis and facilitate hepatic β-oxidation, fatty acid uptake by the liver is enhanced in response to fasting [19,20]. In fasted *Trib3*$^{-/-}$ mice, the hepatic mRNA levels of CD36, a plasma membrane fatty acid translocase, are comparable to wild type mice; however, the expression of L-FABP, a liver-specific intracellular fatty acid trafficking protein, is elevated in the livers of *Trib3*-deficient mice, relative to wild type, following 48 h of fasting (Fig. 2A).

To make the energy stored in fatty acids readily available to other tissues, ketone bodies are produced by the liver and released into the bloodstream [19,20]. HMGCS2, the rate-limiting enzyme of hepatic ketogenesis, is transcriptionally upregulated in the fasting murine liver [20], and demonstrates a higher level of expression in 48-h-starved *Trib3*$^{-/-}$ mice compared to *Trib3*$^{+/+}$ mice (Fig. 2A). In a further adaptation to carbohydrate deficiency, the liver serves as the major site of gluconeogenesis [19,20], and gluconeogenic enzymes are upregulated by fasting [18,20]. However, in contrast to the ketogenic HMGCS2, the expression levels of the two analyzed gluconeogenesis enzymes (PEPCK and G6P) are similar in *Trib3*$^{+/+}$ and *Trib3*$^{-/-}$ mice at all studied time-points (Fig. 2A). The liver also produces lipoprotein particles, which mediate the transfer of fatty acids to extrahepatic tissues for utilization [19,20]. As depicted in Fig. 2A, the expression level of ApoB, an integral component of liver-derived lipoprotein particles, is elevated in *Trib3*$^{-/-}$ mice compared to the wild type, following 48 h of starvation.