Tribbles-1: a novel regulator of hepatic lipid metabolism in humans

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Abstract

The protein tribbles-1, encoded by the gene *TRIB1*, is increasingly recognized as a major regulator of multiple cellular and physiological processes in humans. Recent human genetic studies, as well as molecular biological approaches, have implicated this intriguing protein in the aetiology of multiple human diseases, including myeloid leukaemia, Crohn's disease, non-alcoholic fatty liver disease (NAFLD), dyslipidaemia and coronary artery disease (CAD). Genome-wide association studies (GWAS) have repeatedly identified variants at the genomic *TRIB1* locus as being significantly associated with multiple plasma lipid traits and cardiovascular disease (CVD) in humans. The involvement of *TRIB1* in hepatic lipid metabolism has been validated through viral-mediated hepatic overexpression of the gene in mice; increasing levels of *TRIB1* decreased plasma lipids in a dose-dependent manner. Additional studies have implicated *TRIB1* in the regulation of hepatic lipogenesis and NAFLD. The exact mechanisms of *TRIB1* regulation of both plasma lipids and hepatic lipogenesis remain undetermined, although multiple signalling pathways and transcription factors have been implicated in tribbles-1 function. Recent reports have been aimed at developing *TRIB1*-based lipid therapeutics. In summary, tribbles-1 is an important modulator of human energy metabolism and metabolic syndromes and worthy of future studies aimed at investigating its potential as a therapeutic target.

Introduction

The tribbles family of proteins are being recognized as modulators of many fundamental signalling pathways, biological processes and disease pathologies [1,2]. As the term suggests, this family of pseudokinase proteins is characterized by a distinct lack of kinase activity [3]. However, the past 15 years of research have revealed a myriad of other active functions for these as yet poorly understood proteins. Beyond participating in the regulation of fundamental cellular processes, such as cell cycle progression and proliferation, tribbles proteins are increasingly recognized as potential therapeutic targets. A great deal of prior research has centred on the role of tribbles-1 (*TRIB1*) in the development and progression of leukaemia [4–6], but more recently unbiased human genetic studies have ignited interest in a role for tribbles-1 in human lipoprotein metabolism and cardiovascular disease (CVD) pathogenesis [9,12,13].

Key words: cardiovascular disease, GWAS, lipid metabolism, lipoproteins, Trib1, Tribbles.

Abbreviations: AAV, adeno-associated virus; ALT, alanine transaminase; apoB, apolipoprotein B; C/EBP, CCAAT/enhancer-binding protein; CAD, coronary artery disease; CVD, cardiovascular disease; ERK, extracellular signal-regulated kinase; GLGC, Global Lipids Genetics Consortium; GWAS, Genome-wide association studies; KO, knockout; LAhB, Ldlr KO/Apobec1 KO/human apoB transgenic; LDL, low-density lipoprotein; LDLR, LDL receptor; MAP, mitogen-activated protein; MEK1, mitogen-activated protein kinase kinase 1; NAFLD, non-alcoholic fatty liver disease; Scd1, stearoyl-coenzyme A desaturase1; SNP, single nucleotide polymorphism; TBG, thyroxin-binding globulin; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein; WT, wild-type.

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Genetic associations of *TRIB1* locus with plasma lipids, liver transaminases and coronary artery disease

CVD is the leading cause of death in the developed world [7]. Dyslipidaemia, in particular high plasma levels of lipoproteins containing apolipoprotein B (apoB) as well as high circulating triglyceride (TG) levels, are the most important risk factors for atherosclerotic CVD [8]. This remains the case despite the widespread success of lipid-lowering therapies such as statins and thus there remains a need for novel therapeutics that might further treat dyslipidaemia and CVD in humans. Genome-wide association studies (GWAS) provide an unbiased approach that can potentially identify such novel biological pathways involved in regulation of plasma lipids that might serve as potential therapeutic targets and in recent years much effort has been spent on GWAS to identify loci in the genome associated with plasma lipids and CVD.

Early GWAS of plasma lipid levels in smaller cohorts of humans ($N \cong 10000$) identified a handful of novel genomic loci not previously known to play any role in lipid metabolism. One of these loci exhibiting a significant association with plasma TG levels was the 8q24 locus, with the lead single nucleotide polymorphism (SNP) in these studies falling into a linkage-disequilibrium block that contains the gene *TRIB1* [9]. Subsequent studies replicated this finding [10,11], including a landmark GWAS performed by Global Lipids Genetics Consortium (GLGC), which in 2010 published a GWAS analysis for plasma lipid traits and coronary artery disease (CAD) in > 100000 subjects, yielding

a total of 95 independent loci associated with at least one major lipid trait, more than two-thirds of which are associated with low-density lipoprotein (LDL)-C and/or TG [12]. This study increased the number of novel plasma lipid loci to 59 and of these novel associations only the *TRIB1* locus was associated with all five traits examined: total cholesterol (TC), LDL-C, high-density lipoprotein (HDL)-C, TG and CAD [12]. The most recent GLGC GWAS has identified 157 loci as significantly associated with plasma lipids in humans and *TRIB1* remains one of only four loci associated with all plasma lipid traits examined [13].

Independently, the *TRIB1* locus has been shown by GWAS to be in association with levels of circulating alanine transaminase (ALT) in humans [14]. High circulating ALT levels can be suggestive of hepatocellular damage [15] and may be a surrogate marker for fatty liver [16]. The authors of the GWAS study specifically tested in ∼10000 individuals the association of SNPs in the *TRIB1* region with liver abnormalities identified by computed tomography (CT) scanning that are indicative of hepatic steatosis. Although the *TRIB1* locus did show strong associations with hepatic structural abnormalities, this association did not reach statistical significance after correcting for multiple testing [14]. More recently, researchers in Japan tested the association of three SNPs in the *TRIB1* genomic region with ultrasonographic non-alcoholic fatty liver disease (NAFLD) in ∼5000 Japanese females and saw significant associations between the SNPs and NAFLD [17]. Contrary to this finding, a larger GWAS study aimed at identifying genomic loci associated with NAFLD as ascertained by CT scanning did not find the *TRIB1* locus to be one of the significantly associated genes [18]. These disparate results, however, may be in part due to the difficulty in ascertaining hepatic fat content via non-invasive techniques in large numbers of patients. More highly powered NAFLD GWAS studies are likely to definitively determine the association of *TRIB1* with NAFLD in humans, but currently the evidence strongly suggests that this association does exist.

The association of *TRIB1* with CAD was definitively demonstrated in a separate GWAS performed by the CARDIoGRAMplusC4D consortium in ∼200000 individuals aimed at identifying novel CAD loci [19]. Since the larger GWAS are mainly carried out in humans of European descent, targeted studies have shown that the associations of *TRIB1* with plasma lipids replicate in both African American, as well as Indian populations [20,21].

The preponderance of associations in multiple populations combined with the magnitude of these associations clearly indicates that the genomic region containing *TRIB1* plays a critical role in human lipid metabolism. However, one shortcoming of these studies is that the causal variants in this locus have yet to be identified. Indeed, as part of the 2010 GLGC report, genome-wide significant SNPs were assayed for expression quantitative trait locus (eQTL) association with hepatic transcript levels of nearby genes and no eQTL was identified between *TRIB1* and nearby SNPs despite the GWAS signal lying ∼40 kb downstream of the transcript in the same linkage disequilibrium block as *TRIB1* and with no other gene within 100 kb (Figure 1). A recent report from Douvris et al. [22] found an eQTL between one SNP in the GWAS region and *TRIB1* transcript levels in whole blood from 160 patients. The authors also found that SNPs in the GWAS region affect the transcription of a long non-coding RNA that they dubbed *TRIBAL* and suggested that this may, in part, underlie some of the genetic association in the region. Clearly, this region requires further conditional analyses, fine-mapping and more powerful investigations of SNP–transcript interactions utilizing strategies such as RNA-seq and allele-specific expression, before determining exactly how many genetic signals are present in the region and what the downstream functional effects of the SNPs in these regions are.

In vivo validation of tribbles-1 as a regulator of plasma lipid metabolism

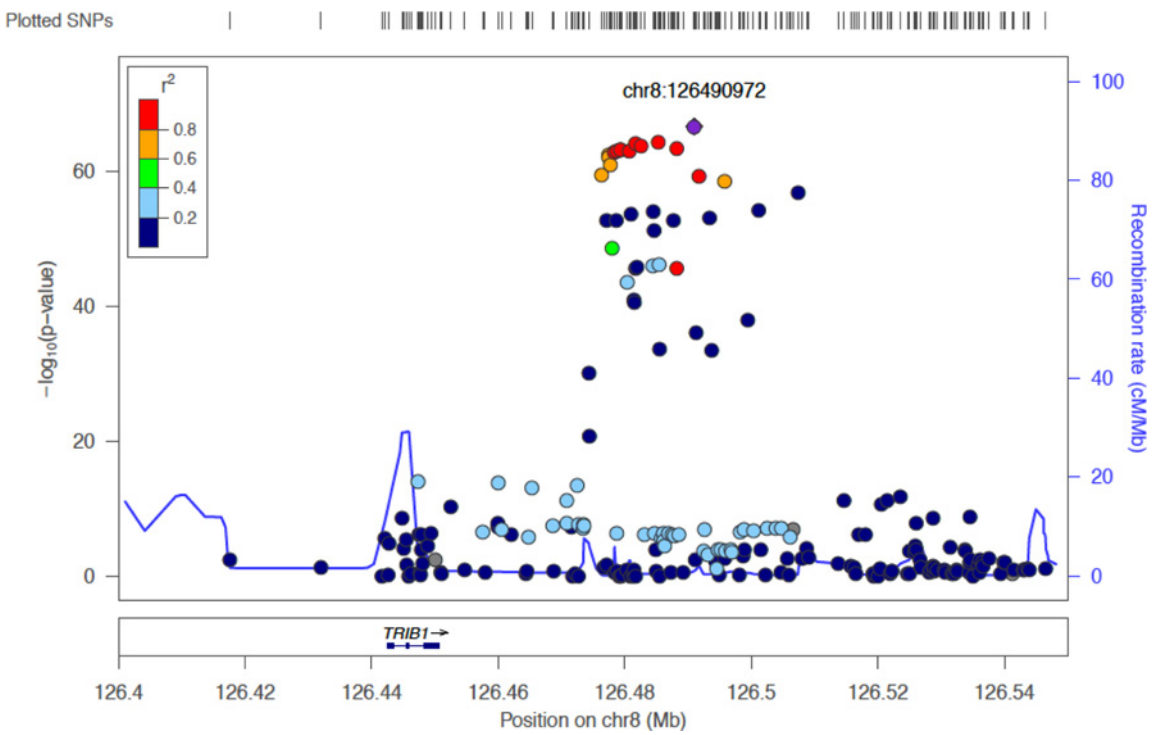
Despite the next closest gene to the 8q24 GWAS signal being >100 kb away, it cannot be simply assumed that *TRIB1* is the causal gene involved in modulating plasma lipid metabolism. Burkhardt et al. [23] utilized an adeno-associated virus (AAV) system to express tribbles-1 *in vivo* via hepatic overexpression of murine *Trib1* in adult C57B/6 mice. The authors cloned the *Trib1* coding sequence in front of the thyroxin-binding globulin (TBG) liver-specific promoter and established stable liver-specific overexpression using AAV serotype 8, known for its high liver specificity and affinity [24]. This overexpression system is ideal for testing the involvement of genes identified by GWAS in hepatic lipid metabolism [25].

The authors observed that overexpression of *Trib1* in the livers of wild–type (WT) mice resulted in reduced levels of plasma cholesterol and TG in a dose-dependent manner. The decreases in cholesterol and TG were present in all lipoprotein fractions examined and FPLC revealed a significant reduction in the very low-density lipoprotein (VLDL)–TG peak, suggestive of a VLDL-specific mechanism of regulation. The authors repeated the *Trib1* overexpression in various mouse models of lipid metabolism, including the LDL receptor (LDLR)-deficient hyperlipidaemic model and the Ldlr KO (knockout)/Apobec1 KO/human apoB transgenic (LAhB) humanized mouse model that has a lipid profile more closely resembling that of humans. In all mouse models tested, *Trib1* overexpression resulted in significant reductions in plasma cholesterol and TG. In the LAhB mice, *Trib1* overexpression caused a significant reduction in plasma apoB protein, the main apolipoprotein component of VLDL and LDL. The investigators also measured many of the same lipid parameters in a previously reported *Trib1* whole-body KO mouse model and saw the expected reciprocal results as compared with the AAV overexpression model.

When the investigators assessed VLDL–TG secretion into the plasma of these mice after treatment with Pluronic-407 (a detergent blocking lipolysis and thus clearance of

Figure 1 | **Association of *TRIB1* region with plasma TG levels**

The 8q24 interval is depicted showing the location of the *TRIB1* gene and the downstream SNPs identified as having significant associations with plasma TGs in humans. The left *y*-axis measures the *P*-value of the SNPs and the colour of each SNPs indicate its r^2-value relative to the lead SNP (rs2954029). Association data to TGs is from the 2013 GLGC study [13] and the figure was generated using LocusZoom [44].



plasma TG), they found that mice treated with hepatic overexpression of *Trib1* had decreased TG secretion. Primary hepatocytes from these mice exhibited not only decreased TG secretion into the media, but also decreased cellular TG content. Indeed when the investigators examined the hepatic transcription of lipogenic genes, they found reduced expression of key genes in the fatty acid synthetic pathway such as acetyl-CoA carboxylase1 (*Acc1*), fatty acid synthase (*Fasn*) and stearoyl-coenzyme A desaturase1 (*Scd1*), among others. Primary hepatocytes expressing tribbles-1 were thus proven to have reduced *de novo* lipogenesis. Thus a model was formed that increased *Trib1* expression can reduce lipogenesis and inhibit VLDL secretion, perhaps through insufficient lipidation of nascent apoB protein.

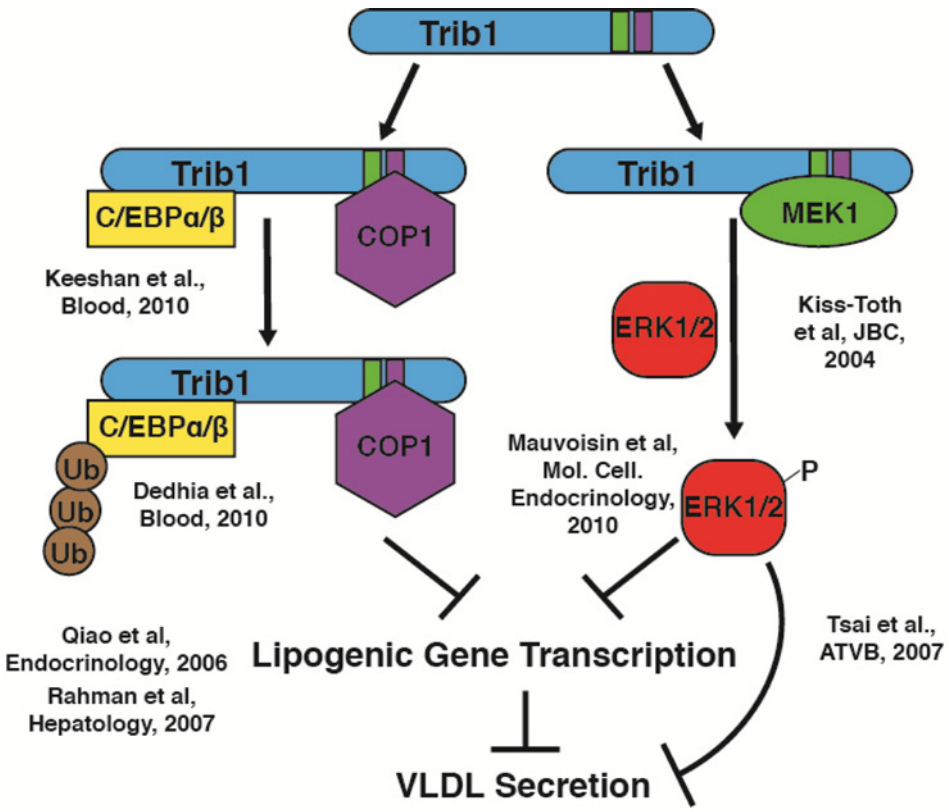
Mechanisms of *TRIB1* regulation of lipid metabolism

Although the work of Burkhardt et al. [23] helped shed light on the physiological roles of *Trib1* in lipid metabolism, the exact molecular mechanism governing this regulation remains to be elucidated. Tribbles-1 has two well-defined functions in the literature: (1) facilitating the ubiquitination of the transcription factor CCAAT/enhancer-binding protein α (*C/EBPα*) and thus promoting its degradation [26] and (2) regulating mitogen-activated protein (MAP) kinase signalling by facilitating the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 by the tyrosine/threonine kinase MAP kinase kinase 1 (MEK1) [27]. Both of these pathways have been shown to participate to some degree in the regulation of lipid metabolism (Figure 2).

The tribbles protein was originally identified in a *Drosophila* mutagenesis screen; the *trb* mutation results in defects in cell migration and mitosis during oogenesis [28–30]. These early studies determined that the role of Trb in *Drosophila* oogenesis is to promote the proteasomal degradation of String, Twine and Slbo, the latter of which is the *Drosophila* homologue of the human *C/EBPα* gene [31]. Subsequent work in the myeloblast 32D cell line has shown that tribbles-1 and tribbles-2 induce the proteasomal degradation of C/EBPα and C/EBPβ by promoting their ubiquitination by the E3 ligase constitutively photomorphogenic 1 (COP1) through direct binding to both the targets and the ligase [26,32]. C/EBPα has long been recognized as important for energy homoeostasis since Wang et al. [33] reported in 1995 that neonates with whole body deletion of C/EBPα died perinatally due to lack of glycogen and hypoglycaemia. These neonates also exhibited a distinct lack of lipids in their hepatocytes and adipocytes. Prior studies have implicated

Figure 2 | Proposed mechanisms for tribbles-1 regulation of plasma lipids in humans

Previous work has identified roles for tribbles-1 in regulating both the ubiquitination and the turnover of the transcription factor C/EBPα, as well as the phosphorylation and activation of ERK1/2 by MEK kinase [26,27]. Both of these pathways have the potential to modulate plasma lipids in humans (relevant citations are shown); however, neither has been directly tested in a hepatic setting with modulation of *TRIB1* protein or transcript.



C/EBPα in the regulation of hepatic lipogenesis in mouse models of obesity. Matsusue et al. [34] showed that hepatic deficiency of *C/EBPα* in the leptin-deficient *ob/ob* mouse model of obesity abbrogated fatty liver caused by a high-carbohydrate diet. Additionally, the authors found that mice with hepatic *C/EBPα* deficiency had decreased lipogenic gene expression and decreased transcription of *SREBP1*, a master regulator of fatty acid synthesis. Qiao et al. [35] repeated most of these findings in the diabetic *db/db* mouse model by treating those mice with adenovirus containing siRNA directed to *C/EBPα*. Interestingly *C/EBPα* deficiency in *ob/ob* mice decreased plasma TGs but not TC, whereas in the *db/db* mice there was decreased plasma cholesterol but not plasma TGs. These reports support a role for *C/EBPα* in the regulation of lipid metabolism by *TRIB1*, and indeed this model was recently confirmed by a report from our group elucidating the roles of Trib1 and *C/EBPα* in hepatic lipid metabolism [36].

As previously mentioned, tribbles-1 has been shown to modulate MAP kinase signalling in HeLa and NIH3T3 cells by promoting the phosphorylation of ERK1/2 by MEK1, a finding not yet replicated *in vivo* [27]. To date, there has been no evidence for the direct phosphorylation of a target by tribbles proteins [37]. Phosphorylation of ERK1/2 has previously been shown in HepG2 cells to down-regulate the expression of *Scd1*, one of the lipogenic genes consistently differentially expressed in experimental models of *Trib1* overexpression [38]. Additionally, Tsai et al. [39] showed that inhibition of MEK/ERK signalling using the ERK1/2 inhibitor U0126 corrected a defect in VLDL assembly in HepG2 cells, greatly increasing the secretion of apoB. The directionality of these observations is in agreement with the observed lipid phenotypes of mice with hepatic overexpression of *Trib1*, thus implicating altered MAP kinase signalling as a potential mechanism of metabolic regulation by tribbles-1.

More recently, Ishizuka et al. [17] published a *Trib1* overexpression/knockdown study using adenovirus and these mice had physiological phenotypes consistent with the report from Burkhardt et al. [23]. They also showed that tribbles-1 could regulate both the mRNA and the protein levels of the transcription factor MLX-interacting protein-like (MLXIPL) (also known as carbohydrate-responsive element-binding protein (ChREBP), another

known regulator of lipogenic gene expression [40]. Epitope-tagged tribbles-1 and MLXIPL interacted in both mammalian two-hybrid assays and overexpression pulldown studies in COS7 cells. The authors showed that protein levels of MLXIPL could be restored to WT levels in *Trib1*-overexpressing COS7 cells via treatment with proteasome inhibitors. The authors concluded that tribbles-1 affects lipogenesis through its interaction with MLXIPL. The extent to which all of these proposed mechanisms affect the regulation of lipid metabolism still needs to be dissected, ideally through genetic epistasis experiments in relevant animal models.

Tribbles-1 as a target for novel therapeutics

One of the main hopes for the unbiased genetic studies outlined earlier in this article has been to uncover novel biology pertaining to lipid metabolism that might be exploited for the production of novel therapeutics. Based on the above described research, therapeutics aimed at increasing levels of *TRIB1* message or protein or small molecules that can increase the function of tribbles-1 could positively affect the plasma lipid profile and cardiovascular health of a patient. Indeed, a recent study from the Broad Institute identified a series of benzofuran-based compounds that could up-regulate transcription of *TRIB1* in HepG2 cells [41]. Ultimately, these compounds had wide ranging effects on the lipid metabolism of these cells, many of which were independent of *TRIB1* expression. The push for *TRIB1*-based therapeutics would benefit from a thorough elucidation of the upstream genetic regulators of this gene. Additionally, continued investigation into the important molecular functions of tribbles-1 and the portions of the protein required for such functions may help inform the development of small molecules that increase tribbles-1 function. Nevertheless, this study suggests that both the interest and potential exist for the development of *TRIB1*-based therapeutics.

Conclusions

The tribbles proteins are increasingly being shown to participate in a wide variety of fundamental cellular processes and tribbles-1 is no exception. Tribbles1 has the added appeal of being identified as significantly and reproducibly associated with multiple human pathologies by human genetics. Not only is the *TRIB1* gene associated with lipid traits and CVD but also with Crohn's disease, plasma adiponectin levels (an adipokine linked to obesity) and plasma liver enzyme levels (a surrogate readout for fatty liver) in humans [14,42,43]. It remains unclear why the other tribbles proteins, known to affect metabolic signalling pathways, have not been identified in many of these GWAS. Nonetheless, tribbles-1 is increasingly recognized as a major regulator of human pathologies. Recent work has focused on identifying novel interactions between tribbles-1 and other proteins and it is likely that more of these interactions will be identified as the tool set for studying tribbles-1 expands [17,45]. However, it is important that researchers in the field attempt to dissect which physiological readouts are governed by which specific interactions. Clearly the tribbles-1 protein can significantly affect the normal biology of cells or tissues and thus one of the challenges moving forward will be discerning which of these molecular phenotypes are directly due to the actions of tribbles-1 itself, or are instead downstream of the primary mechanism of tribbles-1 action. These studies will help inform the development of novel therapeutics that take advantage of the fascinating biology governed by tribbles-1.

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