The transcription factor cyclic AMP–responsive element–binding protein H regulates triglyceride metabolism

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Abstract

Here we report that the transcription factor CREB-H is required for the maintenance of normal plasma triglyceride (TG) levels. CREB-H deficient mice displayed hypertriglyceridemia (HTG) secondary to inefficient TG clearance catalyzed by lipoprotein lipase (Lpl), partly due to defective expression of the Lpl coactivators, *Apoc2*, *Apoa4*, and *Apoa5* and concurrent augmentation of the Lpl inhibitor, Apoc3. Multiple nonsynonymous mutations in *CREB3L3* that produced hypomorphic or nonfunctional CREB-H protein were identified in patients with extreme HTG, implicating a critical role for CREB-H in human TG metabolism.

Keywords

lipid metabolism; dyslipidemia; CREB-H; transcription factor

CREB-H is an endoplasmic reticulum (ER)-bound transcription factor that is highly and selectively expressed only in the liver and the small intestine (Supplementary Fig. 1a,b)[1,2]. CREB-H activation requires a sequential cleavage of the precursor protein by Golgi proteases that liberate the mature N-terminal portion of the protein, which localizes to the nucleus to act as a transcriptional transactivator [3,4]. It has been recently demonstrated that

CREB-H mRNA is induced by metabolic cues such as fasting, fatty acids, and PPARα, suggesting that it might participate in nutrient and energy metabolism [5,6].

To gain insight into the role of CREB-H in nutrient metabolism, we measured metabolic parameters of CREB-H deficient *Creb3l3*^{−/−} mice [1]. We found that *Creb3l3*^{−/−} mice had higher plasma TG levels compared to the WT mice (Fig. 1a), suggesting that CREB-H regulates TG metabolism. Plasma concentrations of free fatty acids (FFA) and cholesterol were slightly changed in *Creb3l3*^{−/−} mice (Fig. 1a). Fractionation of plasma lipoproteins by density gradient ultracentrifugation demonstrated that the very low density lipoprotein (VLDL) fraction of *Creb3l3*^{−/−} mice had higher TG content compared to that of WT mice (Fig. 1b). In line with the induction of the processed CREB-H(N) by fasting in the liver (Supplementary Fig. 1c,d), the HTG phenotype of *Creb3l3*^{−/−} mice was exacerbated upon prolonged starvation (Fig. 1c). Fasting-induced hepatic steatosis was also more pronounced in *Creb3l3*^{−/−} mice than in WT (Fig. 1d). Consistent with higher VLDL TG content in *Creb3l3*^{−/−} mice versus WT, Apob level was also higher in *Creb3l3*^{−/−} than in WT mice (Fig. 1e). Notably, Apoc3, an inhibitor of Lpl, was abundant in VLDL particles purified from *Creb3l3*^{−/−} plasma as determined by mass spectrometry and western blot (Fig. 1e,f).

Fasting plasma TG concentration reflects a balance between the production of VLDL from the liver and the clearance of TG-rich lipoproteins from the circulation, governed predominantly by Lpl present on the surface of vascular endothelium in various organs [7]. To determine if CREB-H plays a role in VLDL secretion from the liver, we measured the rate of TG accumulation in plasma after administration of an Lpl inhibitor, tyloxapol. The VLDL secretion rate was not significantly different between WT and *Creb3l3*^{−/−} mice, arguing against a role for CREB-H in VLDL secretion (Fig. 1g). We next examined if CREB-H deficiency led to decreased Lpl-mediated TG clearance. Olive oil gavage resulted in more pronounced HTG in *Creb3l3*^{−/−} mice than in WT (Fig. 1h), suggesting a defect in TG clearance in the absence of CREB-H. *Creb3l3*^{−/−} mice exhibited lower postheparin Lpl activity compared to WT mice (Fig. 1i), despite normal Lpl mRNA levels in adipose tissue and skeletal muscle (Supplementary Fig. 2), suggesting that plasma cofactors for Lpl might be limiting in *Creb3l3*^{−/−} mice. Consistent with this idea, the addition of WT serum as a source of Apoc proteins significantly increased the Lpl activity retained in post-heparin plasma of *Creb3l3*^{−/−} mice (Fig 1i). The potency of *Creb3l3*^{−/−} serum in augmenting Lpl-mediated hydrolysis of triolein substrate was also attenuated compared to that of WT serum (Fig. 1i). Further, transfusion of WT but not *Creb3l3*^{−/−} plasma efficiently reduced TG levels in *Creb3l3*^{−/−} mice (Supplementary Fig. 3a). Collectively, these data establish that the HTG observed in *Creb3l3*^{−/−} mice is a consequence primarily of decreased TG clearance from plasma, rather than increased TG output from the liver.

To identify CREB-H target genes that might contribute to the HTG phenotype of *Creb3l3*^{−/−} mice, we performed microarray analysis of RNAs isolated from WT and *Creb3l3*^{−/−} mouse liver (Supplementary Fig. 2b). qRT-PCR confirmed lower levels in *Creb3l3*^{−/−} than in WT liver of *Fads1*, *Fads2*, *Elovl2*, *Cidec*, *Apoc2* and *Apoa5*, which have been associated with human TG metabolism (Fig. 2a) [8]. Hepatic *Fgf21*, *Apoa4*, and *Elov5*, and *G0s2* levels were also lower in *Creb3l3*^{−/−} than in WT mice. *Fgf21* is induced by fasting and reduces plasma TG level [9]. *Fads* and *Elovl* genes encode a fatty acid desaturase and elongase, respectively, which play important roles in the synthesis of long chain polyunsaturated fatty acids and the regulation of fatty acid metabolism [10]. *G0s2* and *Cidec* are induced in the liver by fasting, and are known to regulate TG hydrolysis, and lipid droplet formation, respectively [11,12]. *Apoa4*, *Apoa5*, *Apoc2* and *Fgf21* were induced in WT liver by fasting, an effect which was abrogated in *Creb3l3*^{−/−} mice (Fig. 2b). *Apoa4* and *Apoc2* are normally highly expressed in mouse small intestine. Intestinal mRNA levels of these genes were also lower in *Creb3l3*^{−/−} compared to WT mice (Supplementary Fig. 2c). Lower plasma Apoa4 protein level in

Creb3l3^{-/-} than in WT mice correlated well with *Apoa4* mRNA levels in the liver and the intestine (Supplementary Fig. 2d). Apoa5 and Apoc2 activate Lpl, whose activity is further augmented by Apoa4, to facilitate the delivery of hydrolyzed fatty acids to peripheral cells and hence to lower plasma TG levels [13,14]. Despite markedly higher VLDL associated-Apoc3 level in *Creb3l3*^{-/-} than in WT mice, its mRNA levels were not significantly different between these two groups (Supplementary Fig. 2e), suggesting post-transcriptional control of Apoc3 by CREB-H. We postulated that the concomitant reduction of Lpl activators, Apoa4, Apoa5, and Apoc2, and induction of Lpl inhibitor, Apoc3 impaired TG clearance, resulting in higher plasma TG levels in *Creb3l3*^{-/-} than in WT mice. Indeed, injection of recombinant Apoc2 effectively reduced plasma TG levels in *Creb3l3*^{-/-} mice (Supplementary Fig. 3b). In contrast, Apoc2 injection only modestly decreased plasma TG levels in WT mice.

To further explore the identity of CREB-H target genes involved in TG metabolism, we generated transgenic mice that over-expressed a constitutively active CREB-H(N) in the liver (Supplementary Fig. 4a). Both the endogenously processed and the transgenic CREB-H(N) were expressed as 42 kDa proteins in the liver (Supplementary Fig. 4b). Hepatic CREB-H(N) protein levels were 14-fold higher in the transgenic mice compared with WT littermates, despite that the transgenic mRNA level was only 1.25-fold higher than the endogenous *Creb3l3* (Supplementary Fig. 4c), suggesting that ~10% of CREB-H protein is basally processed to the mature form in the liver of chow diet fed WT mice. CREB-H(N) overexpression strongly induced *Apoc2*, *Apoa4*, *Fgf21*, and *Cidec* mRNAs (Supplementary Fig. 4d), mirroring the downregulation of these genes in *Creb3l3*^{-/-} mice. Other apolipoprotein genes located within the same gene clusters as *Apoc2* and *Apoa4* on chromosomes 7 and 9, respectively, were unaffected by CREB-H(N) overexpression in the transgenic liver. Notably, plasma TG levels were significantly lower in CREB-H(N) transgenic mice than in WT littermates, in contrast to the hypertriglyceridemic phenotype of *Creb3l3*^{-/-} mice (Supplementary Fig. 4f). CREB-H(N) overexpression by adenoviral infection also decreased plasma TG levels in mice, concomitantly increasing the potency of the serum to stimulate LPL (Supplementary Fig. 4g,h). Transient transfection assays showed that CREB-H strongly transactivated the *Apoa4*, *Apoc2* and *Fgf21* promoters (Supplementary Fig. 4i), indicating transcriptional activation of these genes by CREB-H.

Emerging evidence suggests that complex traits such as cholesterol and TG are contributed by both common and rare genetic variants [8,15]. Common variants in *CREB3L3* were not observed to be associated with TG in recent large genome wide association studies [16]. Since CREB-H deficiency caused a dramatic increase in plasma TG with no other overt abnormalities in mice, we asked whether mutations in *CREB3L3* could similarly contribute to HTG in humans. Hence, we searched for rare mutations in the *CREB3L3* gene in an established clinical cohort with HTG [17]. We sequenced *CREB3L3* coding regions in 449 unrelated HTG individuals of European Caucasian ancestry (TG > 3.37 mM) and normal *LPL*, *APOC2*, and *APOA5* genes, as well as in 238 matched normolipidemic controls (TG < 2.3 mM) [17]. Remarkably, heterozygous nonsynonymous or insertional mutations in *CREB3L3* were identified in 12/449 HTG individuals, but were completely absent from 238 controls ($P = 0.01$, 2-sided Fisher's exact test, Supplementary Table 1). In contrast, synonymous variants had similar frequency in HTG and control groups (13/238 control versus 12/449 HTG, $P = 0.22$). All mutations localize to the N-terminal region of the protein preceding the bZIP domain (Fig. 2c). Three unrelated HTG individuals were heterozygous for the same complex mutation (designated 245fs), which consisted of a G insertion in the first nucleotide of codon 245 together with a A>T point mutation 7 nucleotides downstream (Supplementary Fig. 5a). One HTG individual was heterozygous for the nonsense *CREB3L3* W46X mutation. Eight HTG probands had heterozygous missense mutations, which included two probands with G105R, two with P166L, one with V180M, one with D182N,

and two with E240K mutations (clinical features summarized in Supplementary Table 1). Lipoprotein profiles of the nuclear families of the 4 individuals with *CREB3L3* nonsense mutations showed a significantly elevated mean plasma TG level in 11 mutation carriers compared with 5 non-carrier first-degree relatives (9.67 ± 4.70 vs. 1.66 ± 0.55 mM, $P = 0.021$, Wilcoxon test) (Supplementary Fig. 5b). Finally, low-coverage resequencing of 179 additional multiethnic genomes in the 1000 Genomes project only identified the G105R variant from our sample and one additional variant not observed in our sample [18], suggesting the occurrence of nonsynonymous variants in *CREB3L3* is extremely rare in normolipidemic populations.

Mutant CREB-H proteins encoded by these human variants were functionally evaluated in transactivation assays using luciferase reporters driven by the proximal *Apoa4, Apoc2* or *Fgf21* promoters. The W46X and 245fs mutations precluded translation of the DNA binding and bZIP domains, and as expected, the resulting mutant proteins failed to transactivate any of the reporters (Fig. 2d). The E240K mutation also severely abrogated the induction of all reporters by CREB-H(N) cotransfection. The D182N mutation exhibited modest but reproducible decreases in fold inductions on all reporters, while V180M did so only on the *Apoc2* reporter (Fig. 2d). In contrast, the G105R and P166L mutations did not significantly affect CREB-H transactivation of the promoters tested. The precise effects of these mutations on the function of CREB-H and their contribution, if any, to the elevated TG levels in the carriers remain to be investigated. In summary, we demonstrated that rare heterozygous variants in *CREB3L3* are significantly associated with the polygenic trait of HTG, similar to other established HTG-associated genes such as *LPL* and *APOA5* [17].

In this report, we identify CREB-H as a novel transcription factor that plays a crucial role in TG metabolism. CREB-H is induced by fasting in the liver and required for the expression of a series of genes that play important roles in TG metabolism, which include *Apoa4*, *Apoa5*, *Apoc2*, *Cidec*, *Elovl2*, *Elovl5*, *Fads1*, *Fads2*, *Fgf21*, and *G0s2*. *Creb3l3*$^{-/-}$ mice displayed HTG and a defect in Lpl-mediated TG clearance, which might be ascribed to defective expression of *Apoc2*, *Apoa4* and *Apoa5* coupled with the augmentation of VLDL-associated Apoc3 with contributions from other yet-to-be-identified CREB-H targets. We further discovered hypomorphic or null mutations in *CREB3L3* that were associated with HTG in humans, implicating a critical role for CREB-H in human TG metabolism. The causal relationship between *CREB3L3* variations and HTG remains to be determined. It will be informative to test if mutant mice harboring any *CREB3L3* mutations found in HTG individuals are predisposed to develop HTG. It will also be of interest to investigate the correlation between plasma TG levels and the frequency of *CREB3L3* sequence variations and their effects on CREB-H function in larger and more diverse populations.

CREB-H is structurally related to SREBPs which play major roles in TG and cholesterol biosynthesis in the liver, as they are synthesized as ER-localizing precursor proteins and activated by S1P and S2P proteases in the Golgi apparatus [3,19]. CREB-H is also functionally related to PPARα which plays an important role in FA oxidation in the liver [20], as both genes are induced by fasting and have common targets such as Fgf21, Apoa5 and G0s2. Future studies will address the molecular mechanisms by which CREB-H is activated and regulates TG homeostasis in concert with other transcriptional regulatory proteins. Elucidation of the signaling pathways that activate CREB-H may lead to novel therapeutic targets to treat human dyslipidemias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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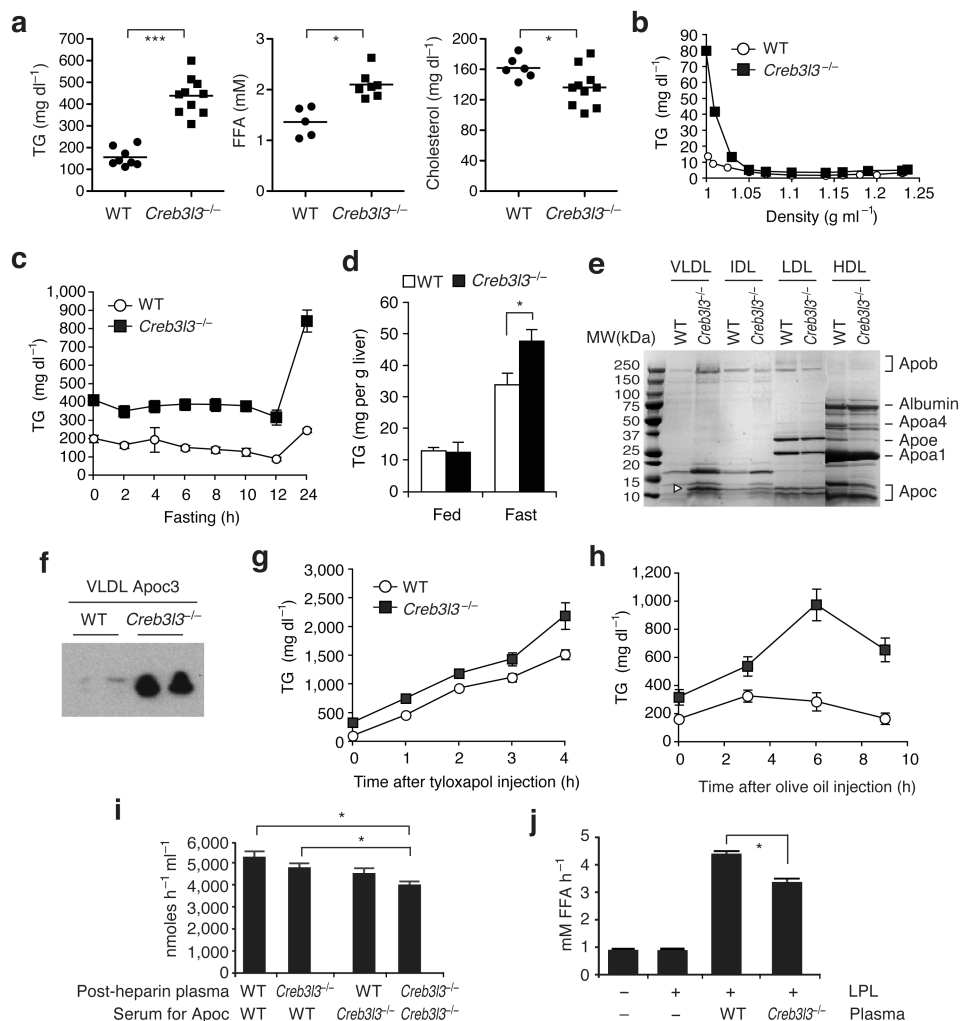
Figure 1. *Creb3l3*⁻/⁻ **mice display HTG secondary to inefficient TG clearance catalyzed by Lpl**
(a)Plasma TG, FFA, and cholesterol levels measured after a 16 h fast. Each dot represents an individual mouse. (b) We separated lipoproteins by density gradient ultracentrifugation of pooled plasma ($n = 3$ per each) after a 24 h fast. TG levels in and density of fractions (1 ml each) sequentially collected from top to bottom. (c) Plasma TG levels in WT ($n = 9$) and *Creb3l3*⁻/⁻ ($n = 7$) male mice deprived of food, starting at 8 AM. (d) Hepatic TG levels measured at fed state or after a 24h fast. $n = 5$ per group. (e) After concentration, fractions from (b) were separated on a 4–20% gradient SDS-polyacrylamide gels. The gel was stained by Coomassie Brilliant Blue G-250. Arrowhead indicates Apoc3 identified by mass spectrometry. VLDL, $d < 1.006$ g ml⁻¹; IDL $d = 1.006$–1.019 g ml⁻¹; LDL, $d = 1.019$–1.063 g ml⁻¹; HDL, $d = 1.063$–1.21 g ml⁻¹. (f) Apoc3 western blot of VLDL fractions. (g) Plasma TG concentrations measured after a 4h fasting followed by *i.v.* injection with tyloxapol (500 mg kg⁻¹). $n = 4$ per group. (h) Plasma TG concentrations measure after a 16 h fasting followed by oral gavage of olive oil (10 ml kg⁻¹). $n = 6$ per group. (i) Post-heparin Lpl activity ($n = 6$ per group) measured in the presence of heat inactivated serum pooled from WT or *Creb3l3*⁻/⁻ mice as Apoc sources. (j) Recombinant LPL was incubated with triolein substrate in the presence of plasma collected from WT or *Creb3l3*⁻/⁻ mice. Values represent FFA concentration released from triolein. $n = 8$ per group. $*P < 0.05$, $***P < 0.0001$, compared to WT mice.
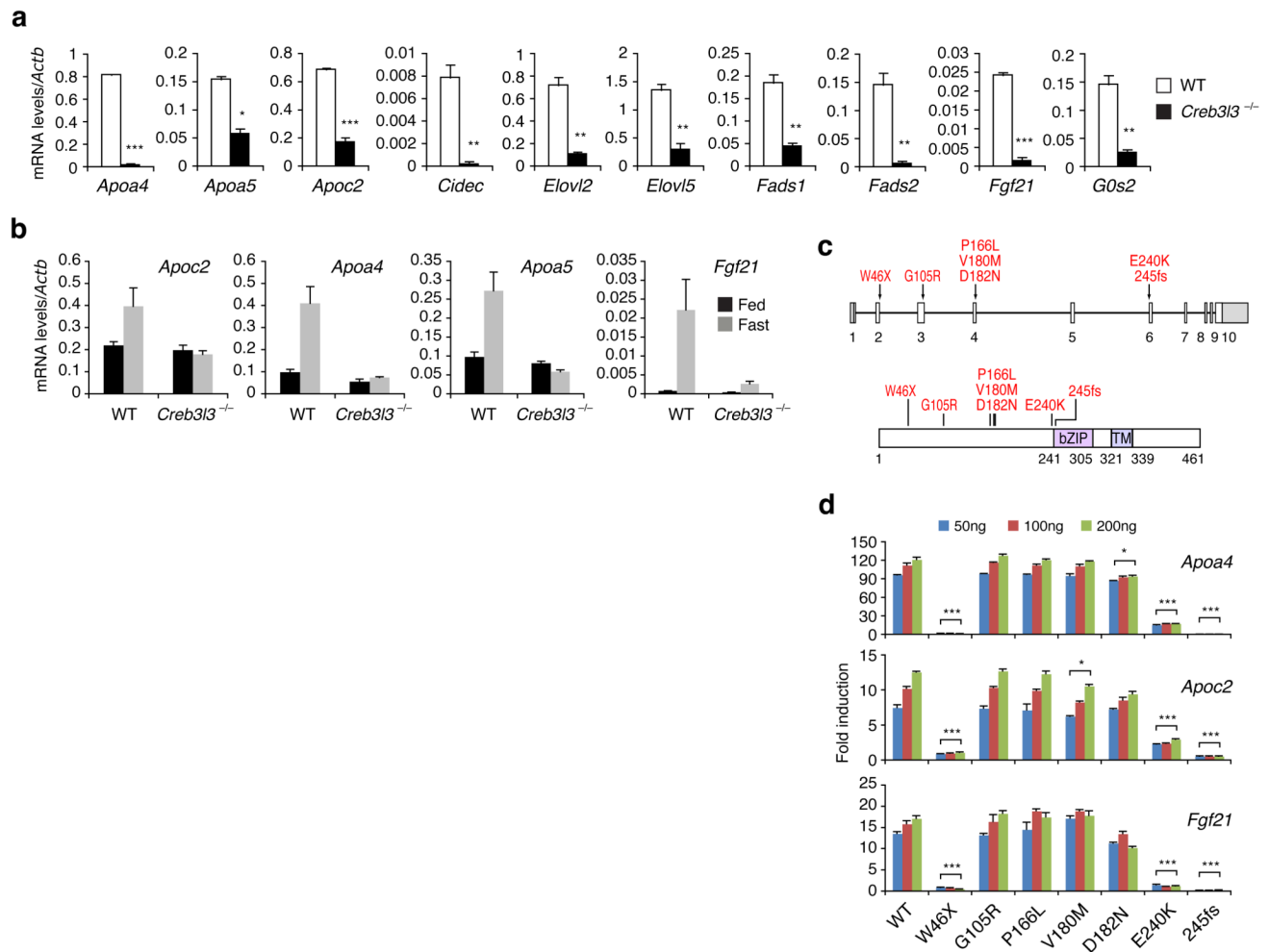
Figure 2. CREB-H controls genes involved in TG metabolism and mutations of *CREB3L3* are associated with human HTG

(a) Hepatic mRNA levels determined by qRT-PCR. Mice were fasted for 24h before sacrifice. $n = 3$ mice per group. Error bars indicate standard deviation. **(b)** *Apoc2*, *Apoa4*, *Apoa5* and *Fgf21* mRNA levels at fed state or after a 24 h fast in WT and *Creb3l3*−/− mice as determined by qRT-PCR. $n = 4$ mice per group. **(c)** Locations of the nonsynonymous mutations found in individuals with HTG, and predicted amino acid changes. bZIP: basic leucine zipper; TM: transmembrane domain. **(d)** Hepa1.6 cells were co-transfected with *Apoa4, Apoc2, or Fgf21* promoterdriven luciferase reporters and the indicated CREB-H(N) constructs. Values represent fold induction of luciferase activities compared to the reporter only transfection. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.