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TRB3 Links the E3 Ubiquitin Ligase COP1 to Lipid Metabolism

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During fasting, increased concentrations of circulating catecholamines promote the mobilization of lipid stores from adipose tissue in part by phosphorylating and inactivating acetyl–coenzyme A carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis. Here, we describe a parallel pathway, in which the pseudokinase Tribbles 3 (TRB3), whose abundance is increased during fasting, stimulates lipolysis by triggering the degradation of ACC in adipose tissue. TRB3 promoted ACC ubiquitination through an association with the E3 ubiquitin ligase constitutive photomorphogenic protein 1 (COP1). Indeed, adipocytes deficient in TRB3 accumulated larger amounts of ACC protein than did wild-type cells. Because transgenic mice expressing TRB3 in adipose tissue are protected from diet-induced obesity due to enhanced fatty acid oxidation, these results demonstrate how phosphorylation and ubiquitination pathways converge on a key regulator of lipid metabolism to maintain energy homeostasis.

Obesity is a major risk factor in the development of type 2 diabetes, which is characterized by a loss of insulin signaling in muscle, liver, pancreatic islets, and adipose tissue (*1*). Mice with a knockout of the insulin receptor (IR) in specific tissues show varying degrees of deterioration in metabolic function (*2*, *3*), but mice with an IR knockout in fat (FIRKO) are phenotypically lean, display enhanced insulin sensitivity in peripheral tissues, and live longer than control littermates (*4*). The mechanism by which loss of insulin signaling in adipose tissue improves metabolic function in FIRKO mice is unclear, but it is thought to be a consequence of enhanced lipolysis and fatty acid oxidation (FAO).

Profiling studies on adipocytes with a knockout of insulin receptor substrate (IRS) genes point to a number of candidate genes that may contribute to the salutary effects of IR or IRS knockouts on metabolic function (*5*). Loss of insulin signaling increases expression of the gene encoding Tribbles 3 (TRB3), a pseudokinase that accumulates in response to fasting and binds to and inhibits the activation of the serine-threonine kinase Akt in the liver (*6*, *7*). Indeed, humans with a gain-of-function mutation in TRB3 have a higher incidence of insulin resistance and diabetes-associated complications (*8*).

TRB3 and its paralogs—TRB1 and TRB2—are catalytically inactive because they lack adenosine 5′-triphosphate (ATP)–binding and catalytic core motifs within their Ser-Thr kinase domains, and they are thought to have evolved instead as adaptor proteins (*9*–*12*). Consistent with this idea, studies in lower organisms have pointed to a potential link between TRBs and the ubiquitin ligase machinery (*9*–*12*). The *Drosophila* protein tribbles, the founding member of this family, coordinates mitosis and morphogenesis during gastrulation by promoting the proteasome-mediated degradation of the G_2-M phase cell cycle regulator String (also called CDC25) (*9*). Tribbles also regulates oogenesis by stimulating the ubiquitination and proteasomal degradation of Slbo, the *Drosophila* homolog of mammalian CCAAT enhancer-binding protein alpha (*11*).

In the process of characterizing TRB3, we found an unexpected role for this pseudokinase in promoting lipid metabolism during fasting through an association with the E3 ligase COP1 (*13*). Confirming its proposed function as an adaptor protein, TRB3 enhanced the COP1-dependent ubiquitination and inactivation of a key regulatory enzyme

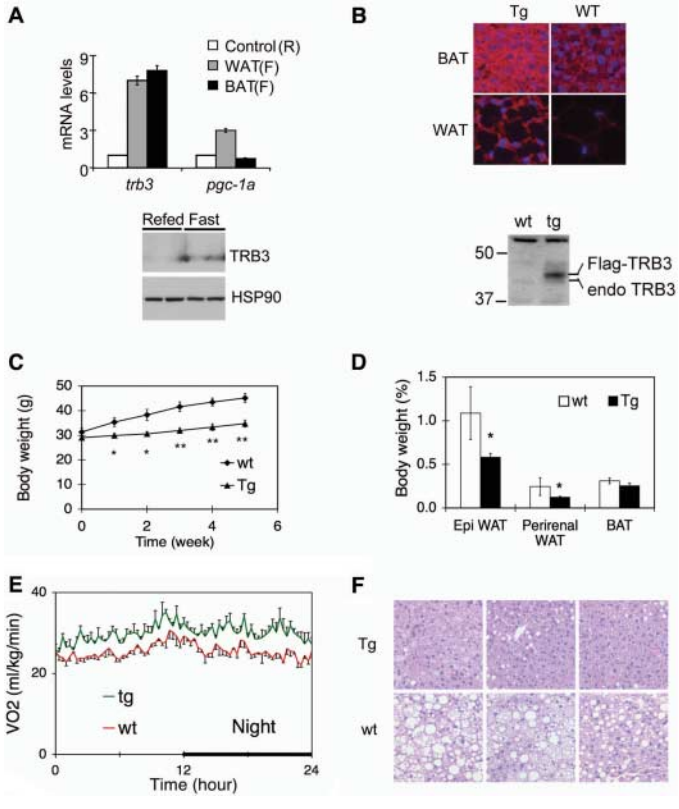

Fig. 1. Protection of transgenic mice expressing TRB3 in adipose tissue from diet-induced obesity. (**A**) (Top) Effect of fasting (F) on amounts of TRB3 and PGC-1α mRNA [fold increase relative to refed (R) levels (set at 1)] in WAT and BAT from wild-type mice. (Bottom) Western blot showing amounts of endogenous TRB3 protein in WAT from wild-type mice under fasting or refed conditions. Amounts of heat shock protein 90 (HSP90) are also shown. (**B**) Immunofluorescence staining (top) and Western blot (bottom) analysis of WAT from overnight-fasted wild-type (wt) littermates and transgenic (Tg) mice expressing TRB3 (F-TRB3) under control of the white and brown adipose-specific aP2 promoter. TRB3 protein was detected with polyclonal antiserum to TRB3. Endogenous (endo) and Flag-tagged TRB3 proteins are indicated. (**C**) Effect of high-fat diet on weight gain in F-TRB3 and control littermates over a 5-week period ($n = 8$) (n, sample size for each cohort). *$P < 0.05$, **$P < 0.01$. (**D**) Relative mass of WAT [epididymal (Epi) or perirenal] and BAT in control and F-TRB3 (Tg) mice, expressed as percentage of total body weight ($n = 3$). (**E**) Relative oxygen consumption (VO_2) of wild-type and F-TRB3 mice over a 24-hour period ($n = 4$). Mice were maintained on a high-fat diet. (**F**) Protection of F-TRB3 mice from hepatic steatosis under high-fat diet conditions. Representative sections from livers of three F-TRB3 transgenic and three wild-type mice on a high-fat diet for 22 weeks. Data in (A) and (C) to (E) are shown as means ± SEM.

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in the fatty acid synthesis pathway during fasting. Our results may help explain the beneficial effects of disrupting insulin signaling in adipose tissue and may also shed light on TRB function in *Drosophila* during development.

Lean phenotype of TRB3 transgenic mice. We generated two independent lines of transgenic mice expressing Flag-tagged TRB3 from the adipose-specific aP2 promoter (F-TRB3 mice) (*14*). In wild-type mice, amounts of TRB3 mRNA and protein in brown and white adipose tissue (BAT and WAT, respectively) were greater in fasting animals than in animals that had been fed after fasting (Fig. 1A). In F-TRB3 mice, TRB3 mRNA and protein levels in WAT and BAT were 2 to 4 times as high as those of wild-type animals under either fasting or feeding conditions and were similar to TRB3 levels in IRS1 (−/−) adipocytes (*5*) (Fig. 1B and fig. S1).

F-TRB3 mice were indistinguishable from control littermates at birth, but gained weight more slowly and had about 10% lower body mass at 8 weeks of age on a normal chow diet (fig. S2). Under high-fat diet conditions, however, F-TRB3 mice showed lower weight gains than did wild-type littermates (Fig. 1C). Epididymal and perirenal fat pads were each reduced in weight by about 50% in F-TRB3 mice, whereas BAT mass did not significantly decrease (Fig. 1D). Histologically, BAT and WAT tissues from F-TRB3 animals had comparable cellularity to those of wild-type littermates, but average adipocyte size was lower in F-TRB3 adipose tissues, suggesting that the metabolism of fat is enhanced in these mice (figs. S3 and S4).

We conducted metabolic studies to determine why adiposity is reduced in F-TRB3 mice. Although physical activity was comparable between both groups, F-TRB3 mice actually consumed 20% more calories than did their wild-type littermates (fig. S5). Under high-fat diet conditions, F-TRB3 mice showed increased O_2 consumption [31.04 ± 0.30 ml O_2/kg/min for F-TRB3 mice versus 26.22 ± 0.32 ml O_2/kg/min for wild-type mice, where the error is SD (Fig. 1E)] as well as a persistent increase in core body temperatures (fig. S5) indicating that F-TRB3 mice are lean in part because of increased energy expenditure.

A high-fat diet is thought to trigger insulin resistance in part by promoting lipid accumulation in the liver (hepatic steatosis). Hepatic steatosis was readily observed in control littermates on a high-fat diet; but lipid accumulation was less pronounced in livers of F-TRB3 mice (Fig. 1F). Correspondingly, F-TRB3 animals exhibited lower circulating concentrations of glucose and insulin (fig. S5), and they appeared to be more sensitive to insulin than were wild-type littermates in an insulin tolerance test (fig. S6).

In view of their increased energy expenditure, F-TRB3 mice could be protected from hepatic steatosis through increased metabolism of fat. Supporting this idea, basal rates of FAO in BAT and WAT were 1.5 to 2 times as high in animals fed normal chow and 3 times as high in animals on a high-fat diet when F-TRB3 mice were compared with control littermates (Fig. 2A and fig. S7). Correspondingly, increased expression of genes encoding enzymes that function in FAO was observed in WAT and BAT from F-TRB3 mice. mRNAs for key transcriptional regulators [peroxisome proliferator-activated receptor α (PPARα), PPARδ, and PPAR gamma coactivator (PGC)–1α] and FAO enzymes carnitine palmitoyltransferase 1 were more abundant in F-TRB3 animals (Fig. 2B). Consistent with the increased core body temperatures, amounts of uncoupling protein 1 (UCP1) mRNA were nearly 14 times as high in WAT from F-TRB3 relative to those in wild-type mice, indicating that TRB3 may protect against diet-induced obesity by enhancing rates of fatty acid oxidation and dissipating this energy in part through thermogenesis.

TRB inhibits ACC activity. We attempted to identify cellular proteins that associate with TRB3 by performing mass spectrometry analysis on endogenous proteins coimmunoprecipitated with Flag-tagged TRB3 in transfected human embryonic kidney (HEK293T) cells. The protein complex was digested with trypsin and directly analyzed with the use of multidimensional liquid chromatography–tandem mass spectrometry together with the SEQUEST database searching software [multidimensional protein identification technology (MudPIT)] (*15*). We identified acetyl-coenzyme carboxylase 1 (ACC1), the rate-limiting enzyme in fatty acid synthesis (*16*) (fig. S8). The TRB3-ACC interaction was also observed in transfected HEK293T cells with the use of epitope-tagged TRB3 and ACC1 polypeptides (fig. S9), and association of the native proteins was detected in WAT of wild-type mice under fasting conditions but not after postfast feeding (Fig. 2C). Other enzymes in the fatty acid synthesis pathway, such as fatty acid synthase (FASN), did not interact with TRB3.

Similar to F-TRB3 mice, mice with a knockout of the *ACC2* gene exhibit a lean phenotype and are resistant to diet-induced obesity, in part because of an increase in fatty acid oxidation in adipose tissue (*17*, *18*). We therefore tested whether the metabolic ef-

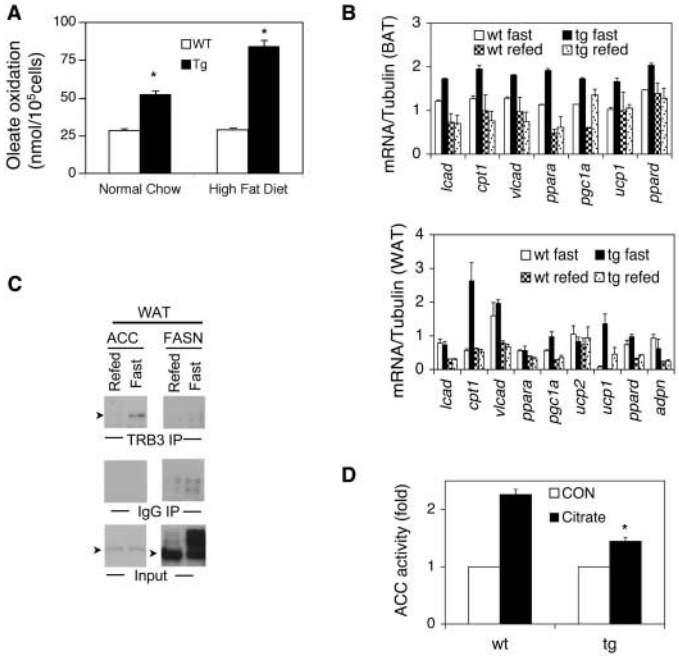

Fig. 2. Effects of TRB3 to promote fatty acid oxidation and disrupt ACC activity. (**A**) Fatty acid oxidation was measured in primary brown adipocytes from refed wild-type and F-TRB3 mice fed a normal chow or high-fat diet. BAT was isolated, and fatty acid oxidation was measured in adipocytes cultured for 12 hours in Roswell Park Memorial Institute (RPMI)–1640 medium containing 0.6 mM oleate (2.5 μCi/ml, [9,10-^{3}H]). (**B**) Quantitative real-time fluorescence polymerase chain reaction analysis of mRNAs for fatty acid oxidation and thermogenic genes in BAT (top) and WAT (bottom) from F-TRB3 and wild-type littermates maintained on a high-fat diet for 22 weeks, under fasting or refed conditions. (**C**) Western blot assay showing recovery of endogenous ACC or FASN from immunoprecipitates (IPs) with antiserum to TRB3 or control rabbit immunoglobulin G (IgG) prepared from WAT of fasted or refed wild-type mice. Input levels (10% of total) of ACC and FASN are shown. (**D**) Fold change in ACC activity [measured as counts per minute (cpm) (^{14}C)/g tissue/min] in WAT from F-TRB3 transgenic mice relative to control littermates. The effect of citrate, an allosteric activator of ACC, is shown. CON, control. Data in (A), (B), and (D) are shown as the means + SEM of three experiments. *$P < 0.05$.

fects of TRB3 in adipose tissue are linked to its association with ACC. In enzymatic assays, ACC activity in WAT from F-TRB3 mice was about one-half that in WAT from normal littermates (Fig. 2D). Infection of 3T3-L1 cells with an adenovirus vector encoding TRB3 also inhibited ACC activity acutely (fig. S10), suggesting that the effects of TRB3 on ACC are likely cell-autonomous.

TRB3 associates with the E3 ubiquitin ligase COP1. Because the *Drosophila* gene *tribbles* (*TRB*) inhibits certain regulatory targets by promoting their proteasomal degradation, we hypothesized that TRB3 might inhibit ACC by a similar mechanism. We performed mass spectroscopy experiments to identify endogenous proteins associated with adenovirus-expressed Flag-tagged TRB3 in 3T3 L1 adipocytes. TRB3 immunoprecipitates contained the E3 ubiquitin ligase constitutive photomorphogenic protein 1 (mCOP1), as well as de-etiolated-1 (DET1) and DNA damage binding protein 1 (DDB1), components of a cullin 4A (Cul4A) ubiquitin ligase (*19*, *20*) (fig. S11). We confirmed the interaction of TRB3 with COP1 in transfected HEK293T cells with the use of epitope-tagged constructs (Fig. 3A). Endogenous TRB3-COP1 complexes were also detected in WAT from fasted mice and at lower levels after postfast feeding (Fig. 3A).

To determine whether TRB3 binds to COP1 and ACC through a direct mechanism, we performed immunoblotting assays with recombinant glutathione *S*-transferase (GST)–TRB3 proteins. Full-length TRB3 bound directly to both ACC1 and COP1 proteins (Fig. 3B), and a truncated TRB3 polypeptide lacking the C-terminal 40 residues (amino acids 315 to 354) associated with ACC1 but not COP1. The C-terminal region of TRB3 contains a sequence similar to the consensus COP1 binding motif ([Asp or Glu] [Asp or Glu]…Val-Pro [Asp or Glu]) (*20*–*22*) that is present in all TRB family members (fig. S13). Mammalian TRB1 also associated with COP1 in transfected HEK293T cells (fig. S14), and a mutant TRB3 polypeptide called Val Pro mutant (VPmt), which contains alanine substitutions at three residues in the COP1 binding motif (Asp^{333}, Val^{336}, and Pro^{337}) was unable to associate with COP1 in transfected HEK293T cells (Fig. 3C).

TRB3 recruits COP1 to ACC. On the basis of its ability to associate with both COP1 and ACC, apparently through distinct surfaces, TRB3 might be expected to mediate an interaction between these proteins and to trigger ubiquitination of ACC1. TRB3 enhanced the interaction of COP1 with ACC1 and ACC2 as detected by coimmunoprecipitation from extracts of transfected HEK293T cells (Fig. 3D and fig. S15). TRB3 also stimulated the ubiquitination of ACC1 and ACC2 by COP1 (Fig. 4A and fig. S15). Confirming the importance of the TRB3:COP1 interaction in this setting, ACC was not ubiquitinated by COP1 in cells expressing COP1 interaction–defective VPmt TRB3 protein (Fig. 4A).

Given our evidence that TRB3 promotes the COP1-dependent ubiquitination of ACC, we tested whether this modification was sufficient to inhibit ACC enzymatic activity. Wild-type TRB3 alone caused a small decrease in ACC1 activity in HEK293T cells, which express small amounts of endogenous COP1 relative to those in 3T3 L1 adipocytes (Fig. 4A). However, ACC activity was reduced to about one-third of that in control cells in cells coexpressing COP1 and TRB3 (Fig. 4A). VPmt TRB3 had no effect on ACC1 activity (Fig. 4A). Taken together, these results suggest that TRB3 may promote loss of fat by mediating the COP1-dependent ubiquitination and inactivation of ACC.

In keeping with the increased abundance of endogenous TRB3 during fasting, amounts of ubiquitinated ACC in WAT were also higher in fasted compared with refed wild-type mice (Fig. 4B). Amounts of ubiquitinated ACC were increased in BAT from F-TRB3 mice compared with those of wild-type littermates (Fig. 4B). Supporting the proposed role of TRB3 as an adaptor protein, amounts of endogenous COP1 associated with endogenous ACC in BAT were higher in F-TRB3 mice than in controls. Amounts of COP1 and ACC associated with TRB3 were also increased in adipose tissue from F-TRB3 mice (fig. S16).

The increased ubiquitination of ACC induced by TRB3 would be predicted to enhance ACC degradation. Under high-fat diet conditions, total amounts of ACC protein in WAT and BAT of F-TRB3 mice were smaller than those in control littermates (Fig. 4C). Conversely, reduction of TRB3 by infection of 3T3-L1 cells with an adenovirus encoding a small interfering RNA against mouse TRB3 induced accumulation of ACC protein and an increase in its enzymatic activity (Fig. 4C and fig. S17), and coexpression of an RNA interference (RNAi)–resistant form of TRB3 protein blocked the induction of ACC by TRB3 RNAi (fig. S18). Reduction of TRB3 in primary cultures of brown adipocytes also increased amounts of ACC protein (Fig. 4C). Amounts of COP1 protein in BAT are much greater than in other tissues such as liver, which may explain in part the distinct metabolic effects of TRB3 in these tissues (Fig. 4C). Taking these results together, we propose that TRB3 may protect against diet-induced

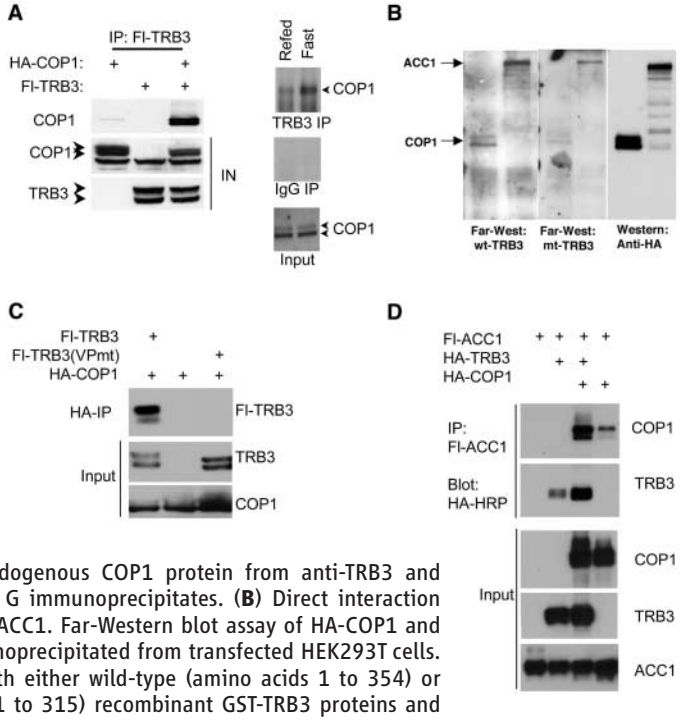

Fig. 3. TRB3-mediated binding of ACC to the E3 ubiquitin ligase COP1. (**A**) (Left) Western blot assay showing recovery of hemagglutinin (HA)–tagged COP1 from immunoprecipitates (IPs) of Flag-TRB3 prepared from transfected HEK293T cells. Input (IN) levels (10%) of each protein indicated. The TRB3 doublet in these studies corresponds to Ser^{51} and Ser^{323} phosphorylated and dephosphorylated forms of the protein (fig. S12). (Right) Western blot assay of WAT extracts from fasted or refed wild-type mice showing recovery of endogenous COP1 protein from anti-TRB3 and control immunoglobulin G immunoprecipitates. (**B**) Direct interaction of TRB3 with COP1 and ACC1. Far-Western blot assay of HA-COP1 and HA-ACC1 proteins immunoprecipitated from transfected HEK293T cells. Blots were incubated with either wild-type (amino acids 1 to 354) or truncated (amino acids 1 to 315) recombinant GST-TRB3 proteins and then developed with antiserum to GST to detect binding of TRB3. The Western blot assay on the right shows immunoprecipitated COP1 and ACC1 proteins. (**C**) Western blot assay comparing the recoveries of wild-type and COP1 interaction–defective VPmt mutant Flag-TRB3 from immunoprecipitates of HA-tagged COP1 prepared from transfected HEK293T cells. (**D**) Effect of TRB3 on association of COP1 with ACC1. Western blot assay shows recovery of HA-tagged TRB3 and HA-tagged COP1 from immunoprecipitates of Flag-tagged ACC1 prepared from transfected HEK293T cells. Input levels (10%) of each protein are shown.

obesity by stimulating fatty acid oxidation in adipose during fasting through the COP1-mediated ubiquitination and degradation of ACC (Fig. 4D).

Parallel fasting pathways. In the fasted state, mammals maintain energy balance by mobilizing triglyceride stores in response to hormonal signals. Triggering of cyclic adenosine 3′, 5′ monophosphate and energy-sensing adenosine monophosphate-dependent protein kinase (AMPK) pathways promote lipolysis and fatty acid oxidation in part by inhibiting ACC, the rate-limiting enzyme in fatty acid synthesis (*16*). ACC activity is modulated acutely by phosphorylation and allosteric regulation and chronically at the levels of biosynthesis and degradation.

Our results indicate that fasting signals also trigger fat metabolism in adipose by increasing amounts of TRB3 protein. Indeed, transgenic mice expressing TRB3 were protected from diet-induced obesity through the disruption of ACC activity and subsequent increase in fatty acid oxidation, metabolic effects similar to those in ACC2-knockout mice (*18*, *23*).

TRB3 appears to inhibit ACC activity by functioning as an adaptor for COP1. The role of TRB3 in this context appears limited, given that we observed no effect of TRB3 on ubiquitination or degradation of other COP1 substrates such as c-Jun and p53 (*19*, *20*). First described in *Arabidopsis* as negative regulators of light-activated genes (*24*–*26*), COP1 and DET1 also modulate genes involved in fatty acid metabolism in plants (*27*). It is possible that the oscillation of COP1 activity with light and dark cycles in plants has its mammalian counterpart in the regulation of metabolic programs during the feeding-to-fasting transition.

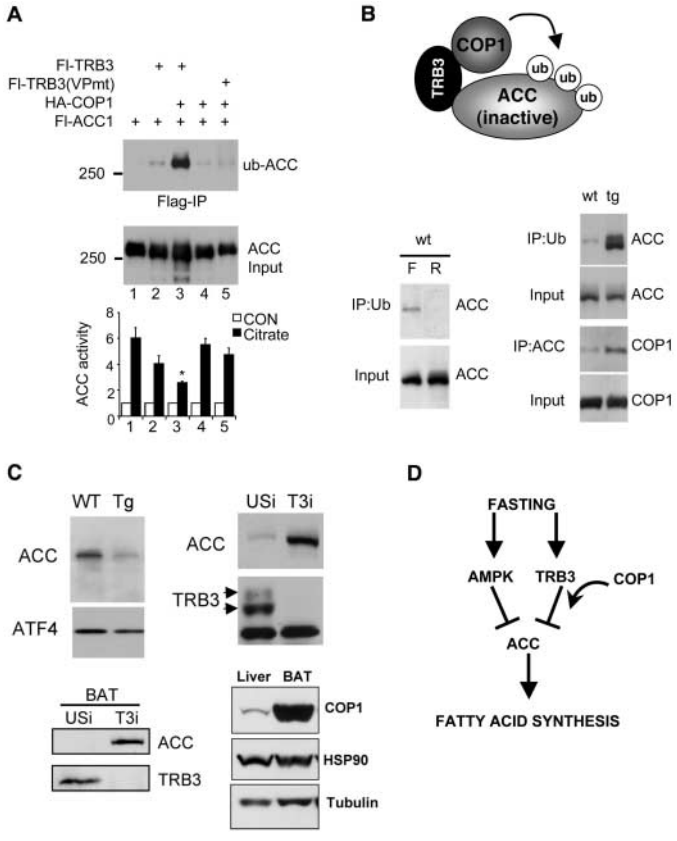

Fig. 4. Effects of TRB3 on ubiquitination (ub) and degradation of ACC by COP1. (**A**) (Top) Effect of overexpression of wild-type and COP1 interaction–defective (VPmt) TRB3 on ubiquitination of ACC1 in HEK293T cells expressing exogenous COP1 as indicated. Amounts of ACC ubiquitination were determined by coexpressing Myc-tagged ubiquitin; top panel shows Western blot of Myc-ubiquitin recovered from immunoprecipitates of Flag-ACC1. Input levels of Flag-tagged ACC (10%) are shown. (Bottom) Effect of wild-type or COP1 interaction–defective (VPmt) TRB3 on >ACC enzymatic activity in HEK293T expressing COP1 in the presence or absence of 2 mM citrate. Numbered sets of bars correspond to numbered lanes in blot above. CON, control. Data are shown as means + SEM. *$P < 0.05$. (**B**) TRB3 promotion of ACC ubiquitination in adipose tissue during fasting. (Left) Western blot assay showing recovery of endogenous ACC from immunoprecipitates with anti-ubiquitin prepared from WAT of wild-type mice under fasting (F) or refed (R) conditions. Input amounts of ACC (10%) are shown. (Right) Relative amounts of ubiquitinated ACC in BAT from wild-type and F-TRB3 transgenic mice. Blot shows amounts of ACC recovered from immunoprecipitates of BAT extracts with anti-ubiquitin. Input levels (10%) of ACC are shown. Below, a Western blot shows the amounts of endogenous COP1 recovered from immunoprecipitates of endogenous ACC prepared from BAT of wild-type and transgenic mice as indicated. (**C**) Promotion of ACC degradation by TRB3. (Top left) Western blot showing amounts of ACC in WAT from control and transgenic littermates under high-fat diet conditions. ATF4, activating transcription factor 4. (Top right) Western blot assay showing TRB3 and ACC protein amounts in control (USi) and Ad-TRB3 RNAi (T3i) expressing 3T3-L1 adipocytes. (Bottom left) Effect of control (USi) and lenti-TRB3 RNAi (T3i) on amounts of TRB3 and ACC protein in primary cultures of brown adipocytes. (Bottom right) Western blot showing relative amounts of COP1 in BAT and liver extracts of wild-type mice. (**D**) Model showing that fasting signals inhibit fatty acid synthesis by means of parallel pathways (AMPK and TRB3) that inactivate ACC by means of phosphorylation- and ubiquitin-dependent mechanisms, respectively. TRB3 promotes ACC ubiquitination by functioning as an adaptor for COP1 during fasting.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5781/1763/DC1
Materials and Methods
Figs. S1 to S18
References

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