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TRB3 Inhibits the Transcriptional Activation of Stress-regulated Genes by a Negative Feedback on the ATF4 Pathway*

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Céline Jousse[‡1], Christiane Deval[‡1], Anne-Catherine Maurin[‡], Laurent Parry[‡], Yoan Chérasse[‡], Cédric Chaveroux[‡], Renaud Lefloch[§], Philippe Lenormand[§], Alain Bruhat[‡], and Pierre Fafournoux[‡2]

From the [‡]UMR 1019, Unité Nutrition Humaine, INRA de Theix, 63122 St Genès Champanelle, France and the [§]Institute of Signaling, Developmental Biology and Cancer Research, CNRS-UMR 6543, Centre Antoine Lacassagne, 06100 Nice, France

The integrated stress response (ISR) is defined as a highly conserved response to several stresses that converge to the induction of the activating transcription factor 4 (ATF4). Because an uncontrolled response may have deleterious effects, cells have elaborated several negative feedback loops that attenuate the ISR. In the present study, we describe how induction of the human homolog of *Drosophila tribbles* (TRB3) attenuates the ISR by a negative feedback mechanism. To investigate the role of TRB3 in the control of the ISR, we used the regulation of gene expression by amino acid limitation as a model. The enhanced production of ATF4 upon amino acid starvation results in the induction of a large number of target genes like CHOP (CAAT/enhancer-binding protein-homologous protein), asparagine synthetase (ASNS), or TRB3. We demonstrate that TRB3 overexpression inhibits the transcriptional induction of CHOP and ASNS whereas TRB3 silencing induces the expression of these genes both under normal and stressed conditions. In addition, transcriptional profiling experiments show that TRB3 affects the expression of many ISR-regulated genes. Our results also suggest that TRB3 and ATF4 belong to the same protein complex bound to the sequence involved in the ATF4-dependent regulation of gene expression by amino acid limitation. Collectively, our data identify TRB3 as a negative feedback regulator of the ATF4-dependent transcription and participates to the fine regulation of the ISR.

Cells have evolved specific signaling pathways to ensure adaptation to stress. For example, the integrated stress response (ISR),[3] which is highly conserved from yeast to mammals, integrates signaling from multiple stress pathways. The ISR acts downstream of the eukaryotic translation initiation factor 2 (eIF2α) phosphorylation (1–4). In mammals, distinct stress signals activate four eIF2α kinases: PKR (activated by double-stranded RNA during viral infection (5)), GCN2 (activated by uncharged tRNAs, adapts cells to amino acid starvation (6, 7)), HRI (activated by heme deficiency (8)) and PERK (activated by protein load in the endoplasmic reticulum, ER stress (9)). The reversible phosphorylation on serine 51 of eIF2α by either of these kinases inhibits initiation of mRNA translation. Although global protein synthesis is inhibited, the translation of specific mRNAs such as ATF4 is strongly induced (4). This transcription factor plays a crucial role for the adaptation to stresses by regulating the expression of many genes. As shown by Harding *et al.* (1), ATF4 controls many genes involved in metabolism and transport of amino acid and in resistance to oxidative stress. In addition, several ATF4 target genes, such as CHOP, are themselves transcription factors that regulate the expression of a set of stress-induced target genes and amplify the signal initiated by the original stress (10–12).

Because an uncontrolled response to stress may have deleterious effects, cells have elaborated a negative feedback loop that attenuates the ISR. Indeed, in response to stresses, the induction of GADD34 mediates eIF2α dephosphorylation and allows protein synthesis to recover to translate the product of induced genes (13, 14). These observations indicate that GADD34 controls a programmed shift from translational repression to stress-induced gene expression and reconciles the apparent contradiction between the translational and transcriptional arms of cellular stress responses.

In a context of amino acid starvation, it has been shown that the expression of many genes is induced via the GCN2/ATF4 pathway. For two genes (CHOP and ASNS), a cis-acting element involved in the transcriptional activation by leucine starvation was well characterized and has been respectively named AARE (amino acid response element) (15) and NSRE (nutrient-sensing response element) (16). Previous data from our laboratory (17) and others (18, 19) have shown that these elements are able to bind ATF4.

Among the mRNA species that are induced by amino acid limitation, the highest induction ratio was obtained for TRB3, the mammalian homologue of *Drosophila tribbles*, also known as TRIB3, NIPK, SINK, or SKIP3. An emerging literature suggests that this ubiquitously expressed protein has a scaffold-like regulatory function for a number of signaling pathways. Several reports described TRB3 as a regulatory protein for p65/RelA

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 33-4-73-62-45-62; Fax: 33-4-73-62-47-55; E-mail: fpierre@clermont.inra.fr.

[3] The abbreviations used are: ISR, integrated stress response; AARE, amino acid response element; ASNS, asparagine synthetase; ATF, activating transcription factor; CHOP, C/EBP homologous protein; MEF, mouse embryonic fibroblast; qRT-PCR, quantitative reverse transcriptase-PCR; NSRE, nutrient sensing response element; DMEM, Dulbecco's modified Eagle's medium; ChIP, chromatin immunoprecipitation assay; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ER, endoplasmic reticulum; DTT, dithiothreitol.

and ATF4. Wu *et al.* (20) showed that TRB3 is critically involved in a negative feedback control pathway of Nf-κB-induced gene expression through the p65/RelA regulation. Another set of publications showed that TRB3 interacts with ATF4 and inhibits the transcriptional activity of overexpressed ATF4 (21, 22). In addition, a recent study supports the potential regulatory role of TRB3 under stressful conditions by demonstrating that cellular stresses can both up- and down-regulate TRB3 expression (23). The functional relevance of TRB3 induction during stress has been studied by Ohoka *et al.* (24) who demonstrated that TRB3 is involved in the coordination of programmed cell death during ER stress. A second line of investigation on TRB3 functions comes from Montminy's laboratory that showed that TRB3 is involved in the control of both glucose and lipid metabolisms (25, 26). Indeed, they show that: 1) TRB3 inhibits Akt activation, 2) its level is elevated during fasting (27), 3) an adenovirus-mediated expression of TRB3 in mice leads to alteration in glucose metabolism. Thus, these authors propose that TRB3 serves as a major modulatory mechanism in the balance of glucose metabolism (25). Recently, the same group demonstrated that TRB3 stimulates lipolysis by triggering the degradation of ACC (acetyl coA carboxylase) in adipose tissue and that transgenic mice overexpressing TRB3 in adipose tissue were protected from diet-induced obesity (26).

Taken together, published data demonstrate that TRB3 is up-regulated by many effectors and is involved in the fine control of several signaling pathways. In the context of amino acid regulation of gene expression, we investigated the role of TRB3 in the regulation of the ISR. We demonstrate that TRB3 is a negative feedback regulator of ATF4-regulated transcription and participates to the fine regulation of the amino acid response pathway and more generally of the ISR.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment Conditions—All cells were cultured at 37 °C/CO_2 5% in Dulbecco's modified Eagle's medium F12 (DMEM F12) (Sigma) containing 10% fetal bovine serum. Parental cell line, *i.e.* HeLa cells overexpressing Tet-repressor, were cultured in presence of 10 μg/ml Blasticidine and tet-TRB3myc cells in presence of 10 μg/ml blasticidine + 100 μg/ml zeocine. When needed, tetracycline was used for 16 h at 0.1 μM. When indicated, DMEM F12 lacking leucine was used. In all experiments involving amino acid starvation, 10% dialyzed calf serum was used. Mouse embryonic fibroblasts (MEF) deficient in ATF4 were kindly given by Dr. D. Ron (NYU, New York) (1).

DNA Transfection and Luciferase Assay—Cells were plated in 12-well dishes and transfected by the calcium phosphate coprecipitation method as described previously (15). Unless otherwise indicated, 1 μg of luciferase plasmid was transfected into the cells along with 0.1 μg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus immediate-early enhancer/promoter region, as an internal control. Cells were then exposed to the precipitate for 16 h, washed twice in phosphate-buffered saline (PBS), and then incubated with DMEM F12 containing 10% calf serum. Twenty-four hours after transfection, cells were amino acid -starved for 16 h. Cells were then harvested and luciferase and β-galactosidase activity was measured as previously described (28). Relative luciferase activity was given as the ratio of relative luciferase unit/relative β-gal unit.

Plasmid Constructions—2xAARE.*TK*.Luciferase and NSRE1 + NSRE2.TK.Luciferase plasmids were generated as previously described (29). pCDNA4.TO.TRB3-FL.Myc.6xHIS plasmid was generated by cloning the full-length coding sequence of TRB3 in frame with the Myc and His tags of the pcDNA4.TO.Myc.6xHIS vector (Invitrogen). pBIND.Gal4-ATF4-(1–85), pBIND. Gal4-ATF4-(1–186) and pBIND.Gal4-ATF4-(86–186) were obtained by inserting ATF4 coding sequence (amino acids 1–85, amino acids 1–186, and amino acids 86–186, respectively), in-frame with the Gal4 coding sequence in the pBIND vector (Promega).

Analysis of Gene Expression Using Real Time RT-PCR—Total RNA was prepared as previously described (30) and treated with DNase I, Amp Grade (Invitrogen) prior to cDNA synthesis. RNA integrity was electrophoretically verified by ethidium bromide staining. RNA (0.5 μg) was reverse-transcribed with 100 units of Superscript II plus RNase H^- Reverse Transcriptase (Invitrogen) using 100 μM random hexamer primers (Amersham Biosciences), according to the manufacturer's instructions. Primers for mouse sequences (used in $ATF4^{+/+}$, $ATF4^{-/-}$ MEFs): *CHOP* (forward primer, 5′-CCTAGCTTGGCTGACAGAGG-3′; reverse primer, 5′-CTGCTCCTTCTCCTTCATGC-3′), ASNS (forward primer, 5′-TACAACCACAAGGCGCTACA-3′; reverse primer, 5′-AAGGGCCTGACTCCATAGGT-3′), TRB3 (forward primer, 5′-CAGGAAGAAACCGTTGGAGTT-3′; reverse primer, 5′-TTGCTCTCGTTCCAAAAGGA-3′) and primers for human sequences (used in HeLa cells and their derivatives): *CHOP* (forward primer, 5′-CAGAACCAGCAGAGGTCACA-3′; reverse primer, 5′-AGCTGTGCCACTTTCCTTTC-3′), *ASNS* (forward primer, 5′-ATCACTGTCGGGATGTACCC-3′; reverse primer, 5′-CTTCAACAGAGTGGCAGCAA-3′), TRB3 (forward primer, 5′-TGGTACCCAGCTCCTCTACG-3′; reverse primer, 5′-GACAAAGCGACACAGCTTGA-3′) were used and yielded PCR products of approximatively 200 bp in size. To control for RNA quality and cDNA synthesis, β-actin mRNA was also amplified (mouse: forward primer, 5′-TACAGCTTCACCACCACAGC-3′; reverse primer 5′-AAGGAAGGCTGGAAAAGAGC-3′; human: forward primer, 5′-CTCGCAGGTCAAGAGCAAG-3′; reverse primer 5′-GACAGCTGCTCCACCTTCTT-3′). Real-time quantitative PCR was carried out using a LightCycler™ System (Roche Applied Science) as described previously (17). Relative results were displayed as relative levels of *CHOP*, *ASNS*, or *TRB3* per β-actin.

Antibodies—The ATF4 sc-200 antibody, purchased from Santa Cruz Biotechnology, Inc, was used for ChIP and immunoblot experiments. The TRB3 antibody was purchased from Calbiochem, the c-Myc antibody was from Sigma, and the β-actin antibody was from Santa Cruz Biotechnology, Inc.

The antibodies used for acetylated histone immunoprecipitation were acetylated histone H3, 06-599 that recognizes acetylated H3 at Lys-9 and Lys-14 and acetylated histone H4, 06-866 that recognizes acetylated H4 at Lys-5, -8, -12, and -16 from Upstate Biotechnology (Charlottesville, VA).

Immunoblot Analysis—Nuclear extracts for the detection of ATF4, mouse TRB3, and Myc-tagged TRB3 were obtained as follow: Cells were lysed for 5 min at 4 °C in Harvest buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM PMSF, protease inhibitor cocktails from Sigma). Intact nuclei were pelleted at 1000 rpm then washed in 500 μl of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktails from Sigma). Nuclei pellets were then lysed 15 min at 4 °C in Buffer C (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktails from Sigma). Whole cell extracts for the detection of human TRB3 were obtained by lysis for 15 min on ice in radioimmune precipitation assay buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, protease inhibitor cocktails from Sigma). 20–50 μg of proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Protein loading was controlled by Ponceau staining. Membranes were incubated in blocking solution (5% nonfat milk powder in PBS, 0.02% Tween) for 1 h at room temperature. The blots were then incubated with primary antibody in blocking solution overnight at 4 °C. Antibodies were diluted according to the manufacturer's instructions. The blots were washed three times in PBS 0.02% Tween and incubated with the appropriate horseradish peroxidase-conjugated antibody (1:10000) from Cell Signaling Technology in blocking buffer for 1 h at room temperature. After three washes in PBS 0.02% Tween, the blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

ChIP Analysis—ChIP analyses were performed according to the protocol of Upstate Biotechnology, Inc. with minor modifications (31). Cells were seeded at 1×10^6/100-mm dish with DMEM F12 and grown for 24 h. Cells were transferred to fresh DMEM F12 12 h before transfer to either complete DMEM F12 or DMEM F12 lacking leucine for the time period indicated in each figure. Protein-DNA cross-link was performed by adding formaldehyde directly to the culture medium to a final concentration of 1% and then stopped 8 min later by the addition of glycine to a final concentration of 0.125 M. Cross-linked chromatin was sonicated using a Vibra cell sonicator (Bioblock Scientific Technology) for ten bursts of 30 s at power 2 with 1 min cooling on ice between each burst. Extracts from 1×10^6 HeLa cells or MEF were incubated with 5 μg of antibody. The antibody-bound complex was precipitated by protein A-agarose beads (Upstate Biotechnology). The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking overnight at 65 °C and purified using a phenol/chloroform extraction and ethanol precipitation. Real-time quantitative PCR was performed by using a LightCycler (Roche Applied Science) and a SYBR-Green-I-containing PCR mix (Qiagen), following the recommendations of the manufacturer. The immunoprecipitated material was quantified relative to a standard curve of HeLa or MEF genomic DNA. Primers used were the following: human *CHOP AARE*, 5′-AAGAGGCTCACGACCGACTA-3′ and 5′-ATGATGCAATGTTTGGCAAC-3′; mouse *CHOP* AARE (amplicon B), 5′-GGGCAGACAAGTTCAGGAAG-3′ and 5′-ATGATGCAATGTTTGGCAAC-3′; mouse *CHOP* amplicon A, 5′-TCCCCAAGAGAACAAACACC-3′, and 5′-CGGACCTGACATTCCTGACT-3′; mouse *CHOP* amplicon C, 5′-TAGAAGGGAGAGGCTGAGCA-3′ and 5′-CCAATGGCTTCCTGTTTCAT-3′; mouse *ASNS* NSRE, 5′-CAGAACACCTCCTGGCTCTC-3′ and 5′-CCGCTTGCCACCTTAGAGT-3′; human *ASNS* NSRE, 5′-AACAAAAGAGCTCCTCCTTGC-3′ and 5′-AGGGATGTGGACAGCTTGAC-3′. The reactions were incubated at 95 °C for 15 min to activate the polymerase, followed by amplification at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 20 s for 45 cycles. After PCR, melting curves were acquired by stepwise increases in the temperature from 65 to 95 °C to ensure that a single product was amplified in the reaction. The results are expressed as the ratio to input DNA. Samples are the means from at least three independent immunoprecipitations.

Sequential ChIP Analysis—The chromatin extraction and the first immunoprecipitation were performed as described in the previous paragraph. The immunoprecipitated samples were incubated for 5 min at 65 °C in elution buffer (0.1 M $NaHCO_3$, 1% SDS), and the supernatants were used for a second immunoprecipitation.

siRNA Preparation and Transfection—siRNA corresponding to human TRB3 mRNA (5′-CGCUGACCGUGAGAGGAAGdTdT-3′ and 5′-CUUCCUCUCACGGUCAGCGdTdT-3′) were designed. A control siRNA (5′-CGCUGACCGUGAAAGAAAGdTdT-3′ and 5′-CUUUCUUUCACGGUCAGCGdTdT-3′) was used as a negative control. siRNA experiments were performed as described previously (17). Cells were plated in 6-well plates and transfected 24 h later with 2 μg of siRNA and 1 μg of luciferase reporter plasmid when applicable, using the calcium phosphate precipitation. 24 h post-transfection, cells are incubated in either DMEM or leucine-free DMEM for 4 or 16 h then harvested either for luciferase assays or for analysis of gene expression using qRT-PCR, as described above.

Microarray Experiments—Total RNA from either MEFs cells ($ATF4^{+/+}$ or $ATF4^{-/-}$ cells), parental cell line or tet-TRB3myc cells were isolated using RNeasy mini kit (Qiagen). cRNA targets were synthesized and hybridized according to the manufacturer's instructions (Agilent). Mouse microarrays were provided from Réseau National des Génopoles (Evry, France) and human microarrays were from Agilent Technology. The slides were scanned with an Affimetrix 428 scanner (Affimetrix, Santa Clara, CA) using appropriate gains on the photomultiplier (PMT) to obtain the highest intensity without saturation. Gene expression values were background corrected and normalized using the Genepix software and Lowess normalization. We used a Student's *t* test with Bonferroni adjustment (with a *p* value of <0.01) and a ratio cut-off of >2 to identify genes differentially expressed by leucine starvation in $ATF4^{+/+}$ and tet-TRB3myc cells.

RESULTS

TRB3 Induction following Amino Acid Starvation Is ATF4-dependent—To better define the role of the GCN2/ATF4 pathway, we screened genes induced by amino acid starvation. In our experimental conditions, TRB3 has been identified as the most induced transcript following amino acid

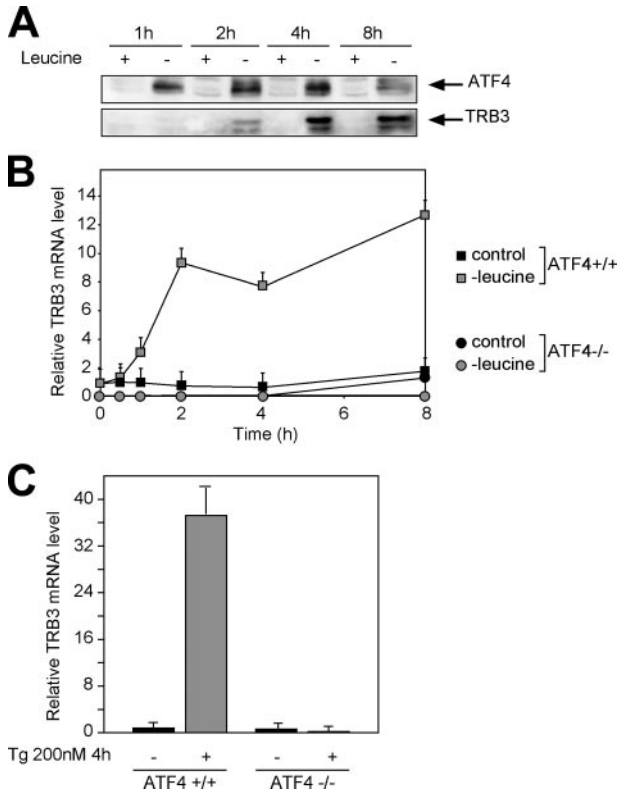

FIGURE 1. **Induction of TRB3 mRNA by leucine starvation.** *A*, MEF cells were incubated either in DMEM/F12 (+leucine) or in DMEM/F12 lacking leucine (−leucine) and harvested after the indicated incubation times. Nuclear extracts were prepared, and immunoblots against ATF4 and TRB3 were performed as described under "Experimental Procedures." *B*, ATF4$^{+/+}$ or ATF4$^{-/-}$ cells were incubated either in DMEM/F12 (*control*, *black dots*, or *squares*) or in DMEM/F12 lacking leucine (−leucine, *gray dots* or *squares*) and harvested after the indicated incubation times. Total RNA was extracted and qRT-PCR were performed as described under "Experimental Procedures." *C*, ATF4$^{+/+}$ or ATF4 $^{-/-}$ cells were incubated in DMEM/F12 containing (*gray bars*) or not (*black bars*) 200 nM thapsigargin, and harvested after 4 h. Total RNA was extracted, and qRT-PCR was performed as described under "Experimental Procedures."

starvation (data not shown). To better characterize the regulation of TRB3 by amino acid starvation, we first analyzed the kinetic of TRB3 expression in response to leucine starvation. Wild-type mouse embryonic fibroblasts (MEFs) were incubated in either DMEM (control medium) or leucine-free DMEM (leucine-free medium) for 1–8 h and the protein level of TRB3 was measured by Western blot. TRB3 was not detected in control cells but was induced following a leucine starvation for 2–8 h (Fig. 1*A*). TRB3 induction by amino acid starvation is not restricted to MEF cells nor to leucine starvation since it was observed in different cell lines and for different essential amino acids limitation (data not shown).

The expression of TRB3 protein was delayed compared with that of ATF4, consistent with the hypothesis that TRB3 is downstream of ATF4. To investigate the dependence of TRB3 toward ATF4, the TRB3 mRNA level was measured by quantitative RT-PCR after leucine starvation, in ATF4$^{+/+}$ or ATF4$^{-/-}$ MEFs (Fig. 1*B*). TRB3 mRNA accumulation was detectable as soon as 1 h after leucine starvation in ATF4$^{+/+}$ cells and reached a plateau after 2 h. In ATF4$^{-/-}$ cells, the steady state level of TRB3 mRNA was undetectable, furthermore no induction was observed following leucine starvation. Because TRB3 induction following amino acid starvation was found to be entirely ATF4-dependent, we checked whether another stress known to induce ATF4, was also able to induce TRB3. For that purpose ATF4$^{+/+}$ or ATF4$^{-/-}$ MEFs were treated for 4 h with 200 nM thapsigargin, a drug causing an ER stress. Thapsigargin also strongly induced TRB3 mRNA in ATF4$^{+/+}$ cells whereas no induction was observed in ATF4$^{-/-}$ cells (Fig. 1*C*).

Overexpression of TRB3 Inhibits CHOP and ASNS Induction by Leucine Starvation—Ord *et al.* (21) have shown that TRB3 interacts with ATF4 and inhibits ATF4 transcriptional activity measured by using reporter gene assays. To investigate the role of TRB3 on the regulation of gene expression by amino acid starvation, a TRB3-inducible cell line was generated. HeLa cells expressing the Tet-repressor were transfected with a plasmid (pCDNA4.TO.TRB3-FL.Myc.6xHIS) containing an in-frame C-terminal fusion of human TRB3 with a peptide encoding the c-*myc* epitope and a polyhistidine (His_6) tag, driven by a hybrid promoter consisting of the human cytomegalovirus immediate-early (CMV) promoter and tetracycline operator 2 (TetO2) sites. The Zeocin-resistant stable cell line obtained was subsequently named "tet-TRB3myc" cell line. Tet-TRB3myc cell line and its parental counterpart were pretreated or not for 16 h with 0.1 μM tetracycline and then incubated in either control or leucine-free medium for 4 h. A Western blot analysis showed that a Myc-tagged-TRB3 was strongly expressed following a tetracycline treatment in the presence or absence of leucine in the tet-TRB3myc cell line, whereas no tagged protein was observed in the parental cell line (Fig. 2*A*). Because tetracycline could interfere with the protein synthesis machinery, we made sure that 0.1 μM tetracycline for 16 h did not affect ATF4 expression in both parental and tet-TRB3myc cells. Finally, using an antibody specific for human TRB3, we showed that the level of TRB3-Myc overexpression upon tetracycline induction and the endogenous TRB3 level observed after amino acid starvation were expressed at the same magnitude (data not shown). This observation indicates that the effects of TRB3 induction are physiologic and not pharmacologic.

To determine whether TRB3 could affect the induction of genes known to be induced by amino acid starvation in a GCN2/ATF4-dependent way, we measured, by quantitative RT-PCR, CHOP and ASNS mRNA levels in tet-TRB3myc cells. In the experiment described in Fig. 2*B*, tet-TRB3myc cells were pretreated or not for 16 h with 0.1 μM tetracycline and then incubated in either control or leucine-free medium for 1–8 h. We found that CHOP mRNA was induced up to 9-fold following leucine starvation in untreated cells, whereas in cells treated with tetracycline (*i.e.* overexpressing TRB3), the induction by leucine starvation was strongly reduced (induction ratio <3). The results were even clearer for ASNS mRNA, whose induction by leucine starvation was completely abolished by TRB3 overexpression. This difference in sensitivity toward TRB3 between CHOP and ASNS could be explained by the existence of other mechanisms that may regulate CHOP expression in response to leucine starvation (*i.e.* mRNA stabilization) (15).

Because the ISR can be turned-on in response to various stimuli, we checked the effects of TRB3 on ASNS expression in cells submitted to an ER stress. Fig. 2*C* shows that ASNS induc-

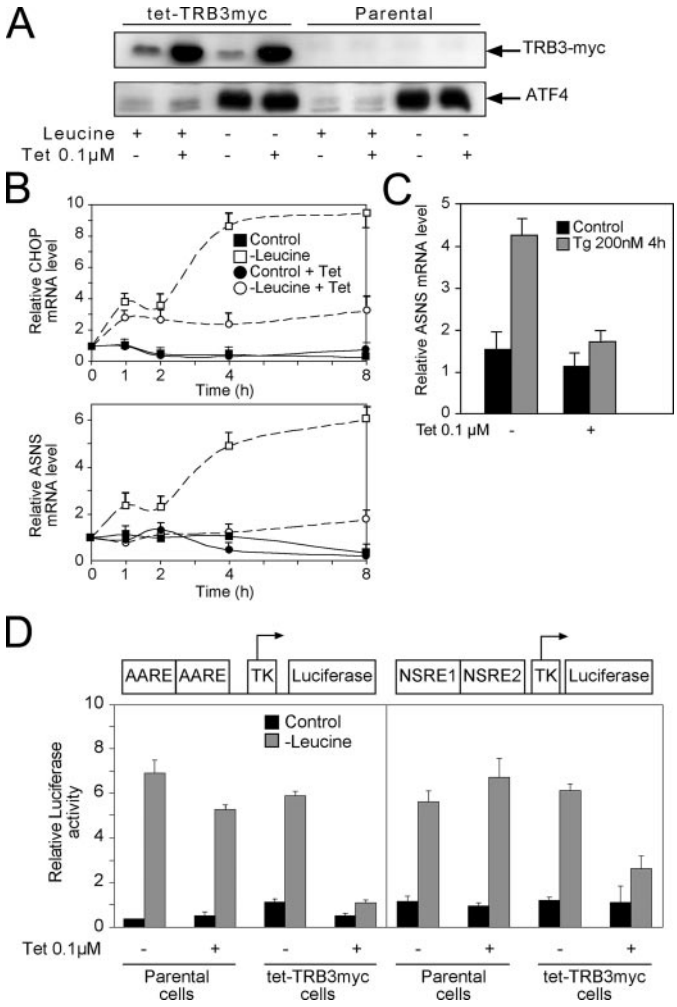

FIGURE 2. **Overexpression of TRB3 inhibits CHOP and ASNS induction by leucine starvation.** *A*, characterization of the tet-TRB3myc cell line: parental or tet-TRB3myc cells were pretreated in DMEM ± tetracycline 0.1 μM for 16 h, then incubated in DMEM/F12 (+leucine) or in DMEM/F12 lacking leucine (−leucine) and harvested after 4 h. Nuclear extracts were prepared and immunoblot against ATF4 (*bottom panel*) and Myc (*top panel*) were performed as described under "Experimental Procedures." *B*, tet-TRB3myc cells were pre-treated in DMEM ± tetracycline 0.1 μM for 16 h (*white* or *black dots*), then incubated in DMEM/F12 (+leucine, *black dots* or *squares*) or in DMEM/F12 lacking leucine (-leucine, *white dots* or *square*) and harvested after the indicated incubation times. Total RNA was extracted, and qRT-PCR was performed as described under "Experimental Procedures." *C*, tet-TRB3myc cells were pretreated in DMEM ± tetracycline 0.1 μM for 16 h, then incubated in DMEM/F12 (control, *black bars*) or in DMEM/F12 containing 200 nM thapsigargin (Tg, *gray bars*) and harvested after 4 h. Total RNA was extracted, and qRT-PCR were performed as described under "Experimental Procedures." *D*, parental or tet-TRB3myc cells were transfected with a reporter plasmid containing either 2 copies of the *CHOP* AARE (−313 to −295) inserted 5′ to the *TK* promoter driving luciferase gene (2xAARE.TK.Luc) or ASNS NSRE1 + NSRE2 inserted 5′ to the *TK* promoter driving luciferase gene (NSRE1 + 2.TK.Luc). Sixteen hours post-transfection, cells were pretreated in DMEM ± tetracycline 0.1 μM for 8 h, then incubated in DMEM/F12 (Control ± Tet 0.1 μM, *black bars*) or in DMEM/F12 lacking leucine (−leucine ± Tet 0.1 μM, *gray bars*) and harvested after 16 h for preparation of cell extracts and determination of luciferase activity as described under "Experimental Procedures."

tion by thapsigargin was abolished when TRB3 was overexpressed. In this experiment we did not measured CHOP expression because, in addition to the ATF4 pathway, ER stress activates other signaling processes regulating CHOP transcription (two ERSE (ER stress element) have been characterized in the CHOP promoter) making the results difficult to interpret. Taken together, the results presented in Fig. 2, *B* and *C* show that TRB3 overexpression inhibits the mRNA accumulation of CHOP and ASNS in response to amino acid starvation or ER stress.

To investigate whether the inhibitory effect of TRB3 acts by regulating the AARE-dependent transcription, we studied the regulation of a luciferase reporter gene driven by a minimum TK promoter flanked by 2 copies of either AARE (2xAARE.TK.Luciferase) or 1 copy of each NSRE (NSRE1 + NSRE2.TK.Luciferase). These constructs were transiently transfected into tet-TRB3myc or into parental cells and the luciferase expression was measured in response to 16-h leucine starvation in the presence or not of tetracycline (Fig. 2*D*). As previously observed, leucine starvation strongly induced both AARE and NSRE1 + 2 transcriptional activity in untreated cells. In contrast, when TRB3 was ectopically induced by tetracycline treatment, little to no induction was observed. These data show that TRB3-dependent inhibition of CHOP and ASNS expression is the consequence of the inhibition of the AARE-dependent transcription.

Inhibition of TRB3 Potentiates ATF4 Target Gene Induction by Leucine Starvation—We formulated the hypothesis that a shut down of TRB3 by RNAi would lead to an overinduction of ATF4 target genes expression upon leucine starvation. First, we checked the efficiency of the TRB3 RNAi by Western blot (Fig. 3*A*) and qRT-PCR (Fig. 3*B*, *first panel*) in control and leucine-starved cells. HeLa cells were transfected either with TRB3 RNAi or Control RNAi, then incubated in either control or leucine-free medium for 16 h. Fig. 3, *A* and *B* shows that TRB3 was strongly induced by leucine starvation in cells treated by Control RNAi, whereas both basal and induced level of TRB3 were strongly diminished in cells treated with TRB3 RNAi. In the same experiment, we then measured ASNS mRNA and found that both basal and induced (leucine starvation) levels of ASNS were increased (respectively 4 and 2 times) when cells were treated with TRB3 RNAi compared with control RNAi.

We then examined the effect of TRB3 RNAi on the AARE-dependent response to leucine starvation using the 2xAARE.TK.Luciferase and NSRE1 + 2.TK.Luciferase constructs. These constructs were transiently transfected into HeLa cells and luciferase measured in control and leucine-starved cells. For both constructs, the basal and the induced (leucine starvation) activities were enhanced when cells were treated with TRB3 RNAi compared with control RNAi (Fig. 3*C*). Taken together these results show that TRB3 is induced by the ISR pathway and represses the ATF4/AARE-dependent transcription. Therefore, TRB3 is a negative feedback regulator of the GCN2/ATF4 pathway.

TRB3 Is Associated with ATF4 in the Protein Complex Bound to the AARE—The molecular mechanism responsible for TRB3-dependent inhibition of the GCN2/ATF4 pathway is not known. One can raise several hypotheses for the mode of TRB3 action. First, upon binding of TRB3 to ATF4, ATF4 might be degraded or destabilized. This hypothesis is coherent with the characteristics of the *Drosophila* TRB3 ortholog Tribble, which has been shown to increase the ubiquitination and degradation of the slbo protein, a C/EBP transcription factor involved in cell migration during oogenesis (32). However, in mammals, TRB3

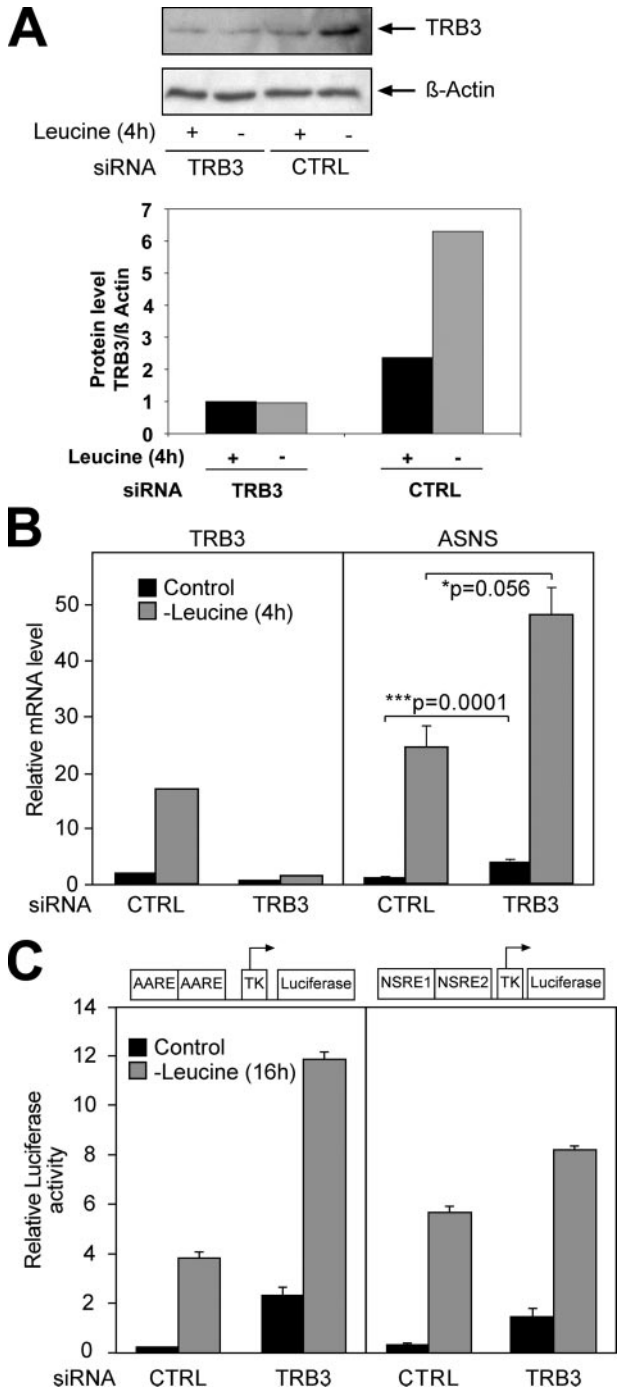

FIGURE 3. **Inhibition of TRB3 potentiates CHOP and ASNS induction by leucine starvation.** *A* and *B*, HeLa cells were transfected with either Control siRNA (*CTRL*) or TRB3 siRNA (*TRB3*). 24 h post-transfection, cells were incubated in either DMEM or leucine-free DMEM for 4 h, then harvested (*A*) for analysis of TRB3, and β-actin protein expression (a quantification of the blot is shown as a histogram) or (*B*) for analysis of gene expression using qRT-PCR, as described under "Experimental Procedures." *C*, HeLa cells were transfected with either Control siRNA (*CTRL*) or TRB3 siRNA (*TRB3*) together with a reporter plasmid containing either 2 copies of the *CHOP* AARE (−313 to −295) inserted 5′ to the *TK* promoter driving luciferase gene (2xAARE.TK.Luc) or ASNS NSRE1 + NSRE2 inserted 5′ to the *TK* promoter driving Luciferase gene (NSRE1 + 2.TK.Luc). 24 h post-transfection, cells were incubated in either DMEM (Control, *black bars*) or leucine-free DMEM (−Leucine, *gray bars*) for 16 h then harvested for preparation of cell extracts and determination of luciferase activity as described under "Experimental Procedures."

does not seem to play a role in protein degradation. Indeed, TRB3 has been shown to interact with CHOP and ATF4 but did not promote their degradation (21, 24). Our results confirm these observations since the elevation of TRB3 in leucine-depleted tet-TRB3myc cells treated with tetracycline did not alter the level of ATF4 (Fig. 2*A*).

Our second hypothesis was that TRB3 might affect ATF4 binding to the AARE. Indeed, leucine starvation triggers ATF4 expression, which then binds to the AARE sequence, in conjunction with other transcription factors, to regulate target genes (17). To determine whether ATF4 binding is affected by TRB3 overexpression, tet-TRB3myc cells were pretreated or not for 16 h with 0.1 μM tetracycline and then incubated in either control or leucine-free medium for 2 h. By using anti-ATF4 ChIP experiments, we found that TRB3 does not affect ATF4 binding on both CHOP AARE and ASNS NSRE (Fig. 4*A*).

Because TRB3 does not prevent ATF4 binding on its DNA *cis*-element, we can hypothesize that TRB3 might bind ATF4 on DNA. To check this hypothesis, cells were incubated for 4 h in either control or leucine-free medium then cells were harvested and ChIP experiment performed using anti-ATF4 and anti-TRB3 antibodies. Firstly, ChIP assays were performed with primer sets covering either the 5′ region (amplicon A), the AARE (amplicon B), or the first intron (amplicon C) of the CHOP gene (Fig. 4*B*). The results show a dramatic increase in both ATF4 and TRB3 binding to the AARE (ampliconB) following leucine deprivation (Fig. 4*C*). Furthermore, binding of ATF4 and TRB3 on the 5′ region and in the first intron of CHOP was very low and not induced by leucine starvation. Taken together these results suggest that ATF4 and TRB3 bind the CHOP gene specifically on the AARE region.

Secondly, to validate that ATF4 and TRB3 belong to the same protein complex that bind the AARE, we performed sequential immunoprecipitations of chromatin with both antibodies (sequential ChIP). A first immunoprecipitation was performed with the anti-TRB3 antibody. After elution, a second immunoprecipitation was carried out with the anti-ATF4 antibody. Appropriate control experiments were performed (no antibody or anti-TRB3 as second antibody). Fig. 4*D* shows that both the CHOP AARE and the ASNS NSRE1 regions are immunoprecipitated from the leucine-deprived cell when anti-TRB3 and anti-ATF4 antibodies were used in the sequential ChIP experiment. These results suggest that TRB3 and ATF4 are present in the same protein complex that binds to the DNA *cis*-response element.

TRB3 Inhibits ATF4-dependent Transcription Independently of ATF4-specific Partners—Because TRB3 inhibits transcription by binding to the ATF4 protein complex bound on DNA, one can speculate about the molecular mechanisms involved in this process. Either it affects chromatin structure (via post-translational regulation of histones) or it interferes with the cross-talk between ATF4 and the transcription apparatus. Chen *et al.* (33) demonstrated a significant change in ASNS promoter histone acetylation during amino acid deprivation (increase in histone H3 and H4 acetylation). Therefore, using ChIP experiments, we investigated the effects of TRB3 overexpression on histone acetylation and found that H3 and H4 acetylation on ASNS promoter observed following leucine star-

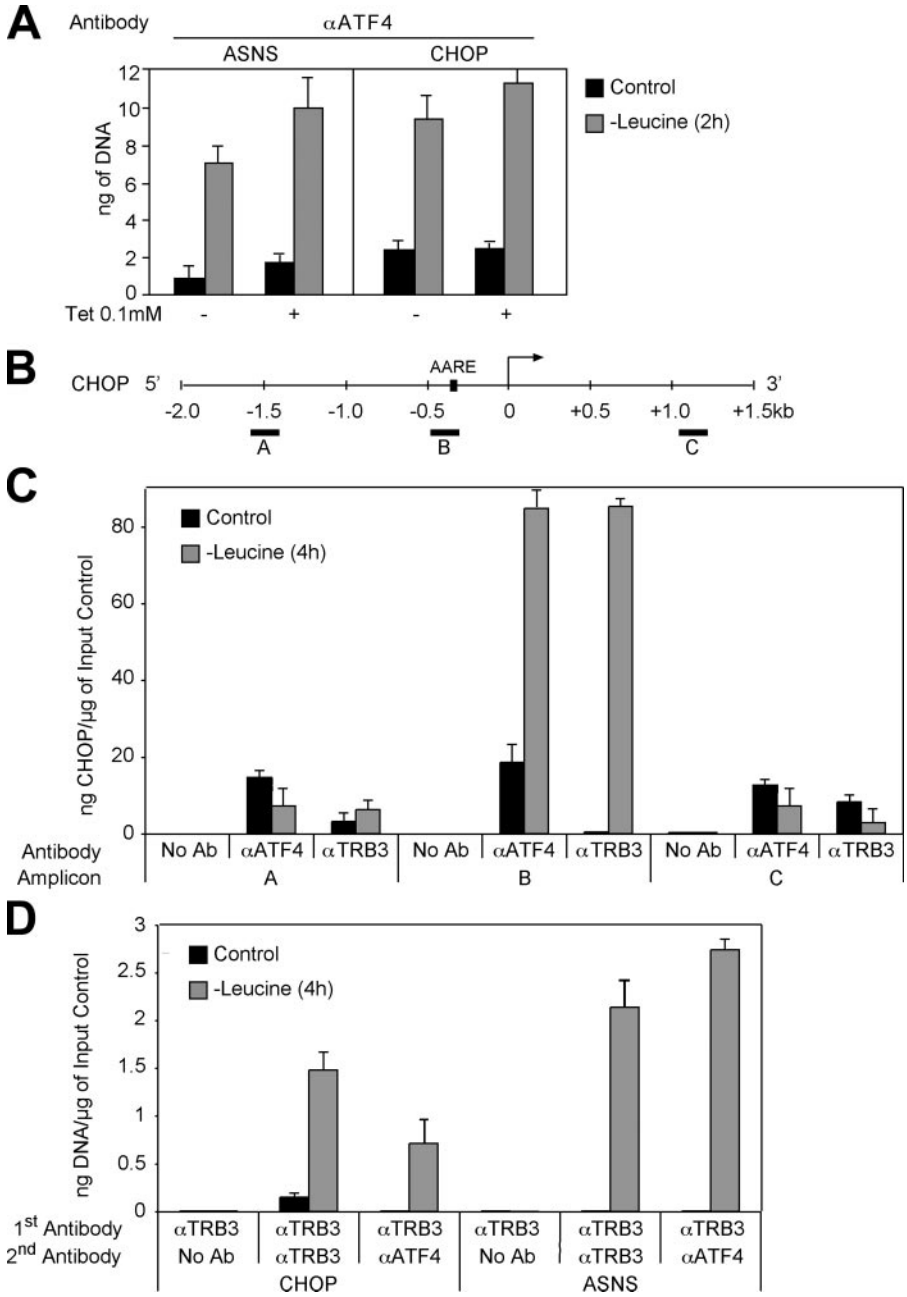

FIGURE 4. **TRB3 is associated with ATF4 in the complex bound to DNA.** *A*, Tet-TRB3myc cells were pretreated in DMEM ± tetracycline 0.1 μM for 16 h, then incubated in DMEM/F12 (Control, *black bars*) or in DMEM/F12 lacking leucine (−leucine, *gray bars*) for 2 h. Chromatin immunoprecipitation analysis was performed as described under "Experimental Procedures" using antibodies specific for ATF4. *B*, scheme of the mouse CHOP gene indicating the different amplicons produced for the ChIP analysis. The AARE is boxed in *gray*. *C* and *D*, MEFs cells were incubated in DMEM/F12 (Control, *black bars*) or in DMEM/F12 lacking leucine (−leucine, *gray bars*) for 4 h. *C*, chromatin immunoprecipitation analysis or (*D*) sequential chromatin immunoprecipitation analysis were performed as described under "Experimental Procedures."

vation were not affected by TRB3 overexpression (data not shown). Similar data were observed with CHOP promoter (not shown). Chromatin structure (*i.e.* modification of histone acetylation) does not seem to be involved in the TRB3-dependent inhibition of ASNS transcription upon amino acid starvation. This finding is coherent with the fact that this inhibition is also observed on exogenous plasmid constructs.

We can then hypothesized that TRB3 could interfere with the cross-talk between ATF4 and some of its partners (either specific or general). Indeed, ATF4 has been shown to interact with general partners such as proteins belonging to the transcription initiation complex such as CBP, TFIIB, TBP (34). In addition, ATF4 is a b-zip transcription factor binding to DNA as a dimer with a specific transcription factor, which can be different according to the context. To test whether specific partners of ATF4 are involved during amino acid starvation, we measured the effect of TRB3 on ATF4-dependent transcription in an artificial context lacking any specific factor. For that purpose, we used a one-hybrid system in which only ATF4 and general factors are present and in which any factor specifically interacting with ATF4 in the context of amino acid starvation are absent. A construct (pBIND.Gal4-ATF4-(1–186)) expressing a fusion protein constituted of the yeast GAL4 DNA-binding domain and the transcriptional activation domain of ATF4 (amino acids 1–186) was co-expressed with a plasmid expressing the luciferase reporter gene under the control of five GAL4 binding sites (pG5-luc). Then we studied the expression of luciferase by ATF4-Gal4 in presence or not of TRB3. Because it was previously shown that TRB3 interacts with ATF4 between the amino acids 50 and 125, we have generated two control constructions (Fig. 5*A*). The first one, (pBIND.Gal4-ATF4-(1–85) expresses the GAL4 DNA binding domain fused to a short domain of ATF4 that maintains a functional transcriptional activation domain but does not interact with TRB3. The second one, (pBIND.Gal4-ATF4-(86–186))contains the domain of ATF4 that interacts with TRB3 and was deleted of the N-terminal region. According to Liang and Hai (34) ATF4 contains several transcriptional activation domains; therefore, this fusion protein should still be able to activate transcription. As expected, Fig. 5 shows that any domain of ATF4 fused to GAL4 resulted in transcriptional activation of the firefly luciferase reporter gene as compared with GAL4 alone (pBIND). Moreover, we found that the concomitant overexpression of TRB3 strongly decreases the transactivation by the GAL4-ATF4-(1–186) and GAL-ATF4-(86–186) constructs whereas it has no effect on the GAL4-ATF4-(1–85)

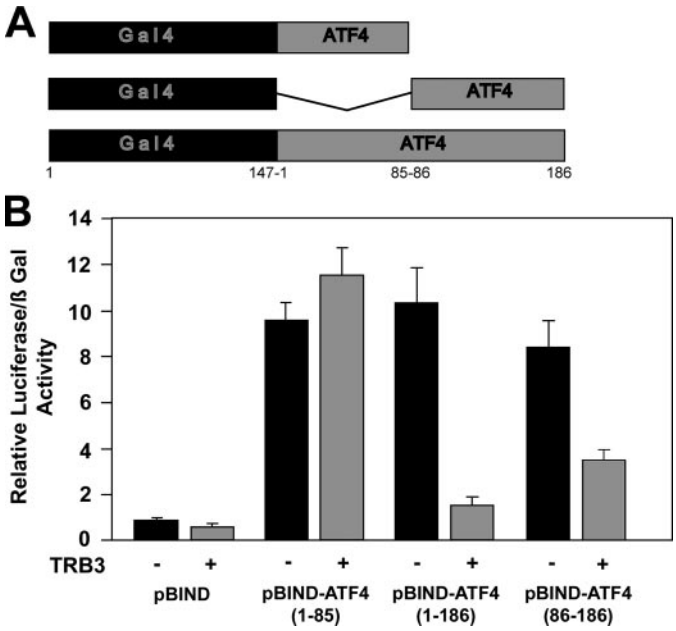

FIGURE 5. **Effect of TRB3 on the transactivating activity of ATF4.** *A*, schematic representation of Gal4-ATF4 fusion constructs used in *B*. *B*, HeLa cells were transfected with a reporter plasmid (pGl5.Luc) containing 5 GAL4 binding sites upstream of the luciferase gene together with pBIND vectors (either pBIND, expressing Gal4; pBIND-ATF4-(1–85), expressing an in-frame fusion of Gal4 with the 85 first amino acids of ATF4; pBIND-ATF4-(1–186), expressing an in-frame fusion of Gal4 with the 186 first amino acids of ATF4 or pBIND-ATF4-(86–186), expressing an in-frame fusion of Gal4 with the amino acids 86 to 186 of ATF4) and an expression vector, pcDNA4 TO Myc His (either empty, *black bars*, or expressing full-length TRB3, *gray bars*). Cells were harvested 40 h post-transfection for preparation of cell extracts and determination of luciferase activity as described under "Experimental Procedures."

construct that cannot bind TRB3 (Fig. 5). These results demonstrate that binding of TRB3 to ATF4 inhibits ATF4 transactivating activity independently of any specific partner. We can then conclude that specific transcription factor partner of ATF4 as well as the chromatin context is not involved in the TRB3 effect. Therefore, it is likely that TRB3 affects the ATF4-dependent transcription by interfering with the association of coactivator(s) or by recruiting corepressor(s) to DNA.

Transcriptional Profiling Shows That TRB3 Affects the Expression of Many Genes Regulated upon the Activation of the GCN2/ATF4 Pathway—It has been previously shown that the ATF4-mediated ISR controls the expression of many genes involved in amino acid metabolism and resistance to oxidative stress in various cell types (1). Using gene expression microarray, we investigated the importance of TRB3 regulatory effects on ATF4-dependent genes. In our study, we used a Student's *t* test with Bonferroni adjustment (with a p value of <0.01 and a ratio cut-off of >2) to identify genes differentially expressed by leucine starvation, and we measured both their dependence toward ATF4 and the effect of TRB3 overexpression on their amino acid-dependent regulation.

First of all, we controlled that tetracycline, by itself, does not regulate gene expression by comparing the profile of genes expressed in parental cells untreated and treated with 0.1 μM tetracycline for 16 h. No genes were found to be down-regulated by tetracycline and few genes were slightly induced. If anything, this could only decrease the importance of the inhibitory effects of TRB3 (not shown). We also checked whether TRB3 overexpression by itself affects the expression of genes by comparing the profile of genes expressed in tet-TRB3myc cells untreated and treated with 0.1 μM tetracycline for 16 h. We found that TRB3 overexpression induces slightly few genes (induction ratio below 2). However, among the genes up-regulated by leucine starvation, none of them was found to be regulated by TRB3 overexpression alone (results not shown).

To determine the dependence toward ATF4 and the effect of TRB3 overexpression, we compared data from $ATF4^{+/+}$ and $ATF4^{-/-}$ cells to data from tet-TRB3myc cells treated or not by tetracycline. Because it was necessary to use two different cell lines (dependence toward TRB3 is determined by using human HeLa cells and dependence toward ATF4 by using MEFs), we restrained the comparison to genes induced by leucine starvation in both models. We found 40 genes induced more than 2-fold by a 4-hour leucine starvation in both cell line (Table 1). Among these 40 genes, 34 are ATF4-dependent genes (Table 1, highlighted in yellow) and 6 genes are totally or partially ATF4-independent (Table 1 highlighted in dark or clear green, respectively). In regard to TRB3 effects, we considered that a gene is not affected by TRB3 overexpression when more than 75% of its induction upon leucine starvation is maintained in cells overexpressing TRB3. Twenty-six genes are not affected by TRB3 overexpression (Table 1 highlighted in purple) whereas the induction ratio of 14 genes is decreased (Table 1 highlighted in sharp yellow).

If we now focus on the ATF4-dependent genes, we found that 41% of the ATF4-dependent genes are affected by TRB3 and 100% of the genes affected by TRB3 are indeed ATF4-dependent genes. On the other hand, all the ATF4-independent genes are unaffected by TRB3. It is noticeable, that the effects of TRB3 would appear underestimated because the induction of certain genes by leucine starvation may be only partially ATF4-dependent rendering the consequence of TRB3 expression difficult to measure. We can conclude that TRB3 plays a broad role in down-regulating a large panel of the ATF4-dependent genes.

DISCUSSION

In this article, we show that TRB3 inhibits via a feed-back mechanism, the ISR. Indeed, TRB3 is induced by ISR (ER stress or amino acid starvation) via an ATF4-dependent pathway, then TRB3 overexpression strongly inhibits the ATF4-dependent transcription. At the molecular level, our results suggest that TRB3 belongs to the ATF4 complex bound on its DNA target and inhibits transcription. In the context of genes regulated by amino acid starvation, transcriptional profiling experiment shows that TRB3 represses numerous ATF4-induced genes.

Our results also demonstrate that TRB3 is involved in the control of the basal level of gene expression under control/unstressed conditions. Indeed, in non-stimulated cells, whereas endogenous TRB3 is expressed at a very low level, its invalidation by siRNA increases about 4-fold the basal level of ASNS (Fig. 4). A more modest increase in the basal level of CHOP expression was also found (data not shown). Therefore, based

TABLE 1

List of genes induced at least 2-fold by amino acid starvation in both tet-TRB3myc and ATF4 +/+ cells

-Fold induction is the mean of the ratio of the hybridization signals in -leucine versus control cells (-leu/ctrl). The dependency of each gene either toward ATF4 or toward TRB3 is color-coded as indicated under the table.

NAME	ID human	ID Mouse	GENE NAME	PROCESS	FOLD INDUCTION ATF4 +/+ -leu/ctrl	ATF4 -/- -leu/ctrl	-TRB3 -leu/ctrl	+TRB3 -leu/ctrl
TRIB3	57761	228775	tribbles homolog 3 (Drosophila)	regulation of transcription, DNA dependent	7.3	1.22	5.79	2.6
amino acid metabolism and transport								
CTH	1491	107869	cystathionase (cystathionine gamma-lyase)	cysteine biosynthesis	6.23	0.66	3.17	1.75
PSPH	5723	100678	phosphoserine phosphatase	L-serine metabolism	3.82	0.61	3.11	1.88
ASNS	440	27053	asparagine synthetase	aspartate family amino acid biosynthesis	3.12	1.1	7.86	3.5
SARS	6301	20226	seryl-tRNA synthetase	tRNA aminoacylation	2.74	1.28	2.25	1.98
GARS	2617	353172	glycyl-tRNA synthetase	tRNA aminoacylation	2.5	0.97	3.00	2.11
NARS	4677	70223	asparaginyl-tRNA synthetase	tRNA aminoacylation	2.5	0.99	2.05	2.03
PSAT1	29968	107272	phosphoserine aminotransferase 1	L-serine metabolism	2.49	1.03	4.14	2.97
WARS	7453	22375	tryptophanyl-tRNA synthetase	tRNA aminoacylation	2.47	1.52	2.01	1.71
SLC3A2	6520	17254	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	amino acid transport	2.42	1.08	2.06	1.78
AARS	16	234734	alanyl-tRNA synthetase	tRNA aminoacylation	2.18	0.89	2.68	2.54
PYCR1	5831	209027	pyrroline-5-carboxylate reductase 1	amino acid biosynthsis	2.16	1.02	2.00	1.78
SHMT2	6472	108037	serine hydroxymethyltransferase 2 (mitochondrial)	L-serine and glycine metabolisme	2.07	0.86	2.11	1.94
regulation of transcription								
DDIT3 (=CHOP)	1649	13198	DNA-damage-inducible transcript 3	regulation of transcription, DNA dependent	6.03	1.58	3.47	1.95
ATF3	467	11910	activating transcription factor 3	regulation of transcription, DNA dependent	5.82	3.21	5.8	5.65
CEBPG	1054	12611	CCAAT/enhancer binding protein (C/EBP), gamma	regulation of transcription, DNA dependent	2.62	1.44	3.91	3.32
SNAI2	6591	20583	snail homolog 2 (Drosophila)	regulation of transcription, DNA dependent	2.55	1.8	2.41	2.61
KLF5	688	12224	Kruppel-like factor 5 (intestinal)	regulation of transcription, DNA dependent	2.34	3.31	3.2	3.48
THRAP2	23389	76199	thyroid hormone receptor associated protein 2	regulation of transcription, DNA dependent	2.23	1.6	2.39	2.73
KLF9	687	16601	Kruppel-like factor 9	regulation of transcription, DNA dependent	2.22	1.59	2.02	2.09
CEBPB	1051	12608	CCAAT/enhancer binding protein (C/EBP), beta	regulation of transcription, DNA dependent	2.00	1.33	4.19	3.36
apoptosis								
GADD45A	1647	13197	growth arrest and DNA-damage-inducible, alpha	cell cycle, negative regulation of protein kinase activity	6.56	2.2	3.66	3.27
DDIT4	54541	74747	DNA-damage-inducible transcript 4	apoptosis	4.25	0.92	2.9	2.07
PMAIP1	5366	58801	phorbol-12-myristate-13-acetate-induced protein 1	induction of apoptosis	2.33	1.32	5.52	3.37
TNFRSF12A	51330	27279	tumor necrosis factor receptor superfamily,member 12A	cell death	2.04	0.93	2.48	2.85
translation								
EIF2S2	8894	67204	eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	translational initiation, protein biosynthesis	2.65	1.32	2.69	2.38
EIF4EBP1	1978	13685	eukaryotic translation initiation factor 4E binding protein 1	regulation of translation	2.46	1.13	2.07	1.98
other								
GDF15	9518	23886	growth differentiation factor 15	receptor signaling pathway	4.93	1.13	5.71	2.21
STC2	8614	20856	stanniocalcin 2	response to nutrient	4.53	1.14	4.05	2.3
MTHFD2	10797	17768	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate	folic acid and derivative biosynthesis	3.00	0.98	3.6	2.5
AREG	374	11839	amphiregulin (schwannoma-derived growth factor)	cell-cell signaling	2.85	1.21	4.04	4.52
CTGF	1490	14219	connective tissue growth factor	regulation of cell growth	2.78	4.1	2.25	2.5
XPOT	11260	73192	exportin, tRNA (nuclear export receptor for tRNAs)	transport	2.38	1.28	2.18	2.1
PTGS2	5743	19225	prostaglandin-endoperoxide synthase 2	fatty acid biosynthsis	2.32	3.02	2.86	3.25
WNT11	7481	22411	wingless-type MMTV integration site family, member 11	cell-cell signaling	2.3	1.11	2.05	1.88
PCK2	5106	74551	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	glucose metabolism	2.27	0.89	3.68	2.12
MID1IP1	58526	68041	MID1 interacting protein 1 (gastrulation specific G12-like (zebrafish))	regulation of microtubule organization	2.24	1.01	2.39	2.89
IL6	3569	16193	interleukin 6 (interferon, beta 2)	cell-cell signaling	2.11	5.63	3.65	3.56
JAG1	182	16449	jagged 1 (Alagille syndrome)	development	2.1	1.59	3.39	3.43
HERPUD1	9709	64209	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	response to unfolded protein	2.07	0.27	3.24	1.7

color codes:
- ATF4-dependent genes
- partially ATF4-independent genes
- totally ATF4-independent genes
- TRB3-dependent genes
- TRB3-independent genes

on the example of these two ATF4-regulated genes, our results demonstrate that TRB3 finely controls the expression level of genes regulated by ATF4 under both stressed and unstressed conditions. Because ATF4 is implicated in many biological processes such as memory formation (35), mammalian development (36, 37) amino acid metabolism, and resistance to oxidative stress (1) we can expect that TRB3 also participates to the fine regulation of these physiological processes.

Although TRB3 is conserved in many species, the molecular mechanisms involved in the TRB3-dependent regulation of gene expression appear to be different between insects and mammals. In *Drosophila*, tribbles has been shown to increase specific protein degradation (32). However, in mammals, TRB3 does not seem to play a role in protein degradation. Indeed, published data (21, 24) and our results indicate that ATF4 degradation does not account for the inhibitory effect of TRB3 on ATF4 transactivation. Rather, our data suggest that TRB3 inhibits ATF4 by binding its N-terminal region directly on the DNA *cis*-element. It is thus likely that TRB3 inhibits ATF4 transactivation by interfering with the association/recruitment of coactivator(s)/corepressor(s) to DNA such as CBP, TBP, TFIIB, and RAP30 (34).

Data from literature described TRB3 as a "scaffold-like regulatory protein" for a number of signaling pathways (38). This characteristic is confirmed by the transcriptional profiling experiment, which demonstrate that overexpression of TRB3 alone does not significantly regulate gene expression. One of the known functions of TRB3 concerns the control of apoptosis. Particularly, Ohoka *et al.* (24) have recently shown that TRB3 is involved in the control of cell death during ER stress. ATF4 and CHOP have been shown to be inducers of apoptosis and p65 generally promotes survival. Because TRB3 have been shown to regulate these factors, it is tempting to speculate that TRB3 may be a decision point between these two cellular responses under stress conditions. Our results, showing that TRB3 inhibits the ISR via a feedback mechanism reinforce the hypothesis that TRB3 could be a sensor for ER stress-induced apoptosis. Particularly, in case of a mild activation of the ISR, the induced TRB3 blocks the expression of ATF4-dependent genes. However, the role of TRB3 appears much more complex because Ohoka *et al.* (24) showed that ectopic TRB3 overexpression increased cell death in response to high concentration of tunicamycin. One can conclude that, in case of intense ER stress, TRB3 could cause apoptosis by a function/mechanism different from the inhibition of ATF4-dependent transcription. Because (i) ER stress regulates at least 3 identified signaling pathways and (ii) apoptosis is regulated by the interaction of several pathways, the precise knowledge of the role of TRB3 in the control of apoptosis remains to be clarified.

At a physiological level, the Montminy group showed new roles for TRB3. They established that TRB3 inhibits Akt activation by insulin in liver (25) and inhibits ACC in adipose tissue (26), suggesting a role of TRB3 in lipid and glucides metabolism. Two observations reinforced their data: (i) TRB3 expression was shown to be strongly affected in the case of obesity or insulin resistance and (ii) a polymorphism in the *TRB3* gene is associated with insulin resistance and cardiovascular risk in humans (39). The mechanisms involved in the regulation of TRB3 expression in liver and adipose tissue have not been studied yet. Because TRB3 is involved in the regulation of many different biological functions in several tissues, it is coherent that many processes regulate its expression. We do not know whether ATF4 (or the ISR) plays a role in the control of TRB3 expression in metabolic tissues.

Recent data provide arguments suggesting that TRB3 is involved in the control of amino acid and protein metabolism: 1) Matsushima *et al.* (40) have shown that TRB3 is a potent regulator of p70S6 kinase (S6K1) activation by insulin; 2) our results showing that TRB3 inhibits ATF4 in response to amino acid starvation bring indirect evidence that TRB3 could be involved in the control of amino acid metabolism. Indeed, considering that ATF4 is implicated in the control of amino acid metabolism and transport (1), we could hypothesize that TRB3 could also be indirectly involved in this process. In addition, we have shown that the GCN2 pathway controls the amino acid homeostasis in omnivores (41). Because ATF4 is the only known pathway downstream of GCN2 it is tempting to speculate that ATF4 and thus TRB3 could also participate to the control of amino acid homeostasis. From all these observations we can hypothesize that TRB3 may be a key regulator of metabolism. However, the precise role of TRB3 in the control of the amino acid metabolism, at a physiological level, remains to be investigated.

It is now clear that following the activation of the integrated stress response pathway, a highly coordinated time-dependent program of molecular events takes place, leading to a fine regulation of transcriptional activation. Our results show that TRB3 regulates the ISR by a negative feedback mechanism. Moreover, the endogenous TRB3 is involved under stressed and normal condition in the fine control of ATF4-regulated genes. Several recent publications demonstrate that TRB3 is an important factor in (i) the response to nutrient starvation, (ii) the glucose metabolism, and (iii) the regulation of cellular functions such as apoptosis. Understanding the role of TRB3 and the regulation of its expression level would improve our comprehension on the control of several signaling pathways. TRB3 may be a potential therapeutic target for metabolic diseases or diseases that involve stress-dependent cell death (neurodegenerative diseases, diabetes).

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