Requirement for translocon-associated protein (TRAP) α in insulin biogenesis

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The mechanistic basis for the biogenesis of peptide hormones and growth factors is poorly understood. Here, we show that the conserved endoplasmic reticulum membrane translocon-associated protein α (TRAPα), also known as signal sequence receptor 1, plays a critical role in the biosynthesis of insulin. Genetic analysis in the nematode Caenorhabditis elegans and biochemical studies in pancreatic β cells reveal that TRAPα deletion impairs preproinsulin translocation while unexpectedly disrupting distal steps in insulin biogenesis including proinsulin processing and secretion. The association of common intrinsic single-nucleotide variants in the human TRAPα gene with susceptibility to type 2 diabetes and pancreatic β cell dysfunction suggests that impairment of preproinsulin translocation and proinsulin trafficking may contribute to the pathogenesis of type 2 diabetes.

INTRODUCTION

The Caenorhabditis elegans DAF-2/insulin receptor (InsR) pathway prevents dauer diapause through a conserved phosphoinositide 3-kinase/Akt pathway that inhibits the FoxO transcription factor DAF-16. In the context of reduced DAF-2/InsR signaling, cytoplasmic DAF-16/FoxO translocates to the nucleus and promotes dauer arrest through transcriptional regulation (1, 2). We have used an unbiased forward genetics approach to identify modifiers of DAF-2/InsR signaling and DAF-16/FoxO activity (3–9). Here, we describe the isolation and characterization of trap-1, which encodes a conserved integral endoplasmic reticulum (ER) membrane protein that modulates C. elegans DAF-2/InsR signaling. Experiments in pancreatic β cells reveal a critical role for the mammalian translocon-associated protein 1 (TRAP-1) ortholog TRAPα/signal sequence receptor 1 (SSR1) in insulin biogenesis. We hypothesize that common variation in TRAPα/SSR1 expression and/or activity may contribute to differences in type 2 diabetes risk in the general population.

RESULTS

In a genetic screen for suppressors of the dauer-constitutive phenotype of an eak-7;akt-1 double-mutant strain that exhibits reduced DAF-2/InsR signaling and increased DAF-16/FoxO activity (3–5, 8), we isolated a strain containing a ~3.5-kb deletion, dpDf665, that spanned the trap-1 gene and three exons of the upstream gene Y71F9AL.1 (Fig. 1A). Three independent trap-1 null mutations (fig. S1) phenocopied the dpDf665 deletion (Fig. 1B), whereas a null mutation in Y71F9AL.1 did not (fig. S2A), indicating that the mutant phenotype is a consequence of trap-1 deletion. Trap-1 mutation suppressed the dauer-constitutive phenotype of mutants with reduced DAF-2/InsR signaling (Fig. 1C) but not the phenotype of mutants with reduced signaling in other pathways that inhibit dauer diapause (fig. S2, B and C) (1, 10). Furthermore, trap-1 mutation impaired the induction of DAF-16/FoxO target genes caused by DAF-2/InsR mutation (Fig. 1, D to F) (11). Thus, TRAP1 promotes dauer arrest by specifically antagonizing the DAF-2/InsR pathway. trap-1 was required for the induction of dauer arrest in wild-type animals by dauer pheromone (fig. S2D) (12), indicating that it promotes dauer arrest in response to physiologically relevant environmental stimuli. trap-1 mutation did not substantially influence organism viability and had a mild effect on the number of eggs laid per animal (fig. S2, E and F).

TRAP1 is orthologous to mammalian TRAPα/SSR1 (henceforth referred to as “TRAPα”), a transmembrane ER protein identified on the basis of its interaction with the preprolactin signal peptide during in vitro protein translocation (fig. S3) (13, 14). TRAPα physically associates with three other conserved ER transmembrane proteins (TRAPβ, TRAPγ, and TRAPδ) to form the TRAP complex (15). A functional single-copy TRAP-1::mCherry fusion protein generated by CRISPR-Cas9–mediated knock-in (Fig. 1C) is expressed in several tissues in C. elegans embryos, larvae, and adult animals (Fig. 2, A to C). Coexpression of TRAP-1::mCherry with the ER protein signal peptide fused to green fluorescent protein (GFP) (16) revealed that endogenous TRAP-1 localizes to the ER (Fig. 2D).

Although the biological function of mammalian TRAPα has not been established, it is thought to play a role in cotranslational ER translocation based on its interaction with the preprolactin signal peptide in vitro (13), its necessity for the in vitro translocation of prion protein (17), and its physical proximity to the Sec61 protein translocation channel (18–22). As the expanded C. elegans insulin-like gene family encodes 40 peptides, some of which enhance dauer arrest by antagonizing DAF-2/InsR signaling (23, 24), we hypothesized that TRAP-1 influences DAF-2/InsR signaling by promoting the ER-based biosynthesis of one or more of these insulin-like negative regulators of DAF-2/InsR (23). If this model is correct, then DAF-2/InsR
mutations that impair receptor function downstream of ligand binding should be resistant to suppression by trap-1 mutation, as these mutant DAF-2/InsR receptors would be refractory to changes in ligand-mediated activity caused by trap-1 mutation. We therefore tested the effect of trap-1 mutation on the dauer-constitutive phenotype of eight distinct daf-2/InsR loss-of-function alleles, seven of which affect amino acid residues that are conserved in the human InsR (table S1) (25).

Whereas trap-1 mutation suppressed the dauer-constitutive phenotype of the daf-2 alleles e1368 and m212 (fig. S4A and table S1), both of which encode receptors with missense mutations in the extracellular ligand-binding domain (25, 26), the dauer-constitutive phenotypes of the other six daf-2 alleles were not affected by trap-1 mutation. The functional consequences of four of these six DAF-2/InsR mutations can be inferred from data on human InsRs with point mutations affecting the corresponding conserved residues (table S1). A heterozygous mutation in the human InsR kinase domain corresponding to daf-2(e1391) (26) was found in a patient with autosomal dominant type A insulin-resistant diabetes mellitus with acanthosis nigricans (IRAN) (27) that results in a severe reduction in InsR in vitro kinase activity and autophosphorylation (28). Another patient with type A IRAN was found to be homozygous for a missense mutation in the InsR extracellular domain corresponding to daf-2(m579) (29) that reduces the affinity of InsR for insulin by three- to fivefold (30). A missense mutation affecting the conserved residue in the human InsR corresponding to the glycine mutated in daf-2(m596) was identified in a patient with insulin resistance and leprechaunism (31) that reduces the amount of InsR present at the plasma membrane by more than 90% (32). Last, human InsR harboring a missense mutation affecting the conserved cysteine mutated in daf-2(m577) (25) exhibits a 100-fold reduction in levels of mature tetrameric receptor present at the cell surface compared to wild-type human InsR (33). The failure of trap-1 mutation to suppress the dauer-constitutive phenotypes of these daf-2/InsR alleles (fig. S4A and table S1) suggests that TRAP-1 may act upstream of DAF-2/InsR and is consistent with a role for TRAP-1 in the biogenesis of insulin-like peptide ligands that antagonize DAF-2/InsR. However, the fact that none of these daf-2/InsR alleles is a null allele precludes drawing firm conclusions from these data about whether TRAP-1 acts at the level of DAF-2/InsR ligands.

To further test the hypothesis that TRAP-1 promotes the biogenesis of antagonistic DAF-2/InsR ligands, we determined the effect of trap-1 mutation on the dauer-constitutive phenotype of animals with mutations in three genes encoding DAF-2/InsR agonist peptides (24). trap-1 mutation partially suppressed the dauer-constitutive phenotype of ins-4 ins-6;daf-28 triple mutants (fig. S4B), consistent with a model whereby TRAP-1 promotes antagonist insulin-like peptide ligand action.

As human insulin antagonizes DAF-2/InsR signaling when it is expressed in C. elegans (23), we explored the possibility that mammalian TRAPα promotes insulin biogenesis. Endogenous TRAPα colocalized with KDEL ER resident proteins but not with the Golgi marker GM130 (34) in rat INS 832/13 pancreatic β cells (Fig. 3A). To assess the role of TRAPα in preproinsulin translocation, we generated INS 832/13 cells lacking TRAPα using CRISPR-Cas9–based genome engineering.
Fig. 3. TRAPα promotes preproinsulin ER translocation, insulin biogenesis, and insulin secretion in INS 832/13 cells. (A) Immunostaining of INS 832/13 cells with anti-TRAPα antibodies reveals colocalization with ER proteins recognized by anti-KDEL antibodies (top) but not with the Golgi protein GM130 (bottom). Nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole). (B) SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and anti-proinsulin immunoblotting of lysates from INS 832/13 wild-type (WT) or TRAPα KO cells. Cells were untreated or treated with the proteasome inhibitor MG132 as indicated. pPI, preproinsulin; PI, proinsulin. (C) SDS-PAGE and anti-proinsulin immunoblotting of lysates from INS 832/13 wild-type or TRAPα KO cells after pretreatment with phosphate-buffered saline (PBS), digitonin (DIG), or Triton X-100 (TRX) and subsequent exposure to Proteinase K (PK). (D) SDS-PAGE and immunoblotting of lysates (left) and anti-TRAPα immunoprecipitates (right) from 293 T cells transfected with either empty vector (EV) or a complementary DNA (cDNA) encoding the A24D MIDY preproinsulin mutant. The arrow denotes preproinsulin present in the anti-TRAPα immunoprecipitate. (E) SDS-PAGE and anti-insulin immunoblotting of lysates from INS 832/13 wild-type and TRAPα KO cells. (F) SDS-PAGE of anti-insulin immunoprecipitates of cell lysates (C) or conditioned media (M) from INS 832/13 wild-type or TRAPα KO cells after pulse-labeling with [35S]Met/Cys and chase for the indicated times. Ins-B, insulin. Quantification of total proinsulin and insulin (C + M) is shown in (G) and (H), respectively. (I) Glucose-stimulated insulin secretion assay on INS 832/13 wild-type, TRAPα KO, and TRAPα KO cells transfected with a TRAPα cDNA. (J) SDS-PAGE of anti-α1-antitrypsin (AAT) immunoprecipitates of cell lysates (C) or conditioned media (M) from INS 832/13 wild-type or TRAPα KO cells after pulse labeling and chase as described for (F). INS 832/13 wild-type or TRAPα KO cells were transfected with a plasmid encoding AAT 48 hours before pulse-labeling. (K) Quantification of total AAT (C + M) at the indicated time points.
editing. Normally, preproinsulin is nearly undetectable by immunoblotting with anti-proinsulin antibodies (Fig. 3B, first two lanes), but TRAPα knockout (KO) resulted in detectable amounts of preproinsulin (Fig. 3B, third lane) that increased further upon pretreatment of cells with the proteasome inhibitor MG132 (Fig. 3B, last lane).

These data could be consistent with a requirement for TRAPα in the translocation of preproinsulin such that in the absence of TRAPα, untranslocated preproinsulin is degraded by the proteasome. Alternatively, the data in Fig. 3B could be consistent with impaired preproinsulin processing by signal peptidase in the ER. To distinguish between a translocation defect and a signal peptidase processing defect in TRAPα KO cells, we determined the protease sensitivity of preproinsulin in TRAPα KO cells after selective permeabilization of the plasma membrane (Fig. 3C). In wild-type cells, preproinsulin was fully translocated into the ER and processed to proinsulin that was protease-resistant after plasma membrane permeabilization with digitonin but degraded after full solubilization of internal membranes with Triton X-100 (Fig. 3C, third and fourth lanes). In TRAPα KO cells, accumulated preproinsulin was protease sensitive both after partial and complete membrane permeabilization (Fig. 3C, seventh and eighth lanes). These data indicate that preproinsulin remains in the cytosol in TRAPα KO cells and implicates TRAPα in the ER translocation of preproinsulin.

As TRAPα was found on the basis of its association with the nascent signal peptide of preprolactin (13), we considered the possibility that TRAPα binds to preproinsulin. Since steady-state levels of wild-type preproinsulin are extremely low due to rapid ER translocation and signal peptide cleavage to generate proinsulin (Fig. 3, B and C), we tested the ability of the A24D mutant preproinsulin to interact physically with endogenous TRAPα. This mutant was first identified in two patients with neonatal diabetes (35). Steady-state levels of A24D mutant preproinsulin are increased compared to wild-type preproinsulin due to impaired signal peptide cleavage (36). A24D preproinsulin was detected in anti-TRAPα immunoprecipitates (Fig. 3D), indicating that TRAPα and A24D preproinsulin physically interact. Thus, TRAPα may play a direct role in preproinsulin ER translocation.

While the steady-state level of proinsulin was not affected by TRAPα KO (Fig. 3, B and C, and fig. S5A), anti-insulin immunoblotting revealed an unexpectedly marked reduction in total insulin content in TRAPα KO cells compared to control cells (Fig. 3E). TRAPα knockdown by RNA interference (RNAi) recapitulated this decrease in steady-state insulin levels (fig. S5B), indicating that this phenotype does not represent an off-target effect of genome editing.

To gain further insight into the role of TRAPα in early events governing insulin biogenesis, we pulse-labeled cells with [35S]-labeled methionine and cysteine ([35S]-Met/Cys) and analyzed insulin biosynthetic intermediates by immunoprecipitation immediately after labeling or after a chase of 60 or 120 min (Fig. 3, F to H). In wild-type cells, proinsulin was efficiently labeled with [35S]-Met/Cys (Fig. 3F, lane 1) and processed into mature insulin (Ins-B; lanes 2 and 4). In addition, both proinsulin and insulin were secreted into the media (M; lanes 3 and 5). Notably, in wild-type cells, proinsulin was not detected, consistent with its rapid translocation and conversion to proinsulin in the ER. In contrast, in TRAPα KO cells, newly synthesized preproinsulin was detectable immediately after labeling (lane 6) but was lost thereafter. While proinsulin was still synthesized (lane 6), its recovery at 60 and 120 min after labeling was diminished (compare lanes 7 and 9 to lane 6). Moreover, proinsulin was neither secreted intact nor processed to mature insulin intracellularly (lanes 7 to 10). Therefore, in addition to impairing preproinsulin ER translocation, TRAPα KO also results in reduced proinsulin stability, with markedly decreased insulin biogenesis and secretion. Consistent with these findings, TRAPα KO cells were defective in both basal and glucose-stimulated insulin secretion (Fig. 3I). Both of these phenotypes were partially rescued by transfection with a TRAPα complementary DNA (cDNA).

To elucidate the contribution of the proteasome to increased proinsulin turnover in TRAPα KO cells, we treated [35S]-Met/Cys–labeled cells with the proteasome inhibitor MG132. MG132 treatment increased intracellular proinsulin levels (fig. SSC, compare lane 13 to lane 11 and lane 17 to lane 15). However, this increase did not rescue defects in proinsulin secretion and processing to mature insulin. Therefore, TRAPα KO impairs the intracellular trafficking of proinsulin and the biogenesis of insulin independent of its effect on proinsulin turnover.

To determine whether the proinsulin trafficking defect observed in TRAPα KO cells was due to a general defect in the secretory pathway, we analyzed the biogenesis and secretion of α1-antitrypsin (AAT) in wild-type and TRAPα KO cells. TRAPα KO affected neither the biogenesis nor the secretion of AAT (Fig. 3, J and K). These findings indicate that the secretory pathway is functionally intact in TRAPα KO cells and also demonstrate that TRAPα promotes the ER translocation and subsequent trafficking of only a subset of secreted proteins.

We also used immunofluorescence to independently examine the consequences of TRAPα KO on insulin biogenesis. In wild-type cells, insulin (Fig. 4A) and proinsulin (Fig. 4B) were readily detectable. However, TRAPα KO resulted in a marked decrease in insulin content (Fig. 4A), although proinsulin was still detectable (Fig. 4B). Reintroducing epitope-tagged TRAPα into TRAPα KO cells rescued insulin production in transfected cells (Fig. 4, C and D), and infection of TRAPα KO cells with adenovirus encoding TRAPα at increasing multiplicities of infection revealed that this rescue was dose dependent (fig. S6).

Our finding that TRAPα activity is required for efficient insulin biogenesis and secretion was unexpected. Defects in proinsulin processing and secretion could be an indirect consequence of TRAPα dysfunction in the ER. In mouse fibroblasts, TRAPα physically associates with misfolded ER-associated degradation (ERAD) substrates (37), suggesting that TRAPα may play a role in reducing ER stress. Thus, in pancreatic β cells, loss of TRAPα may cause abnormalities in proinsulin processing and secretion (Fig. 3, F to H) due to a general increase in ER stress.

To determine whether C. elegans TRAP-1 and mammalian TRAPα play roles in mitigating ER stress, we assayed for constitutive activation of the unfolded protein response in animals and β cells lacking TRAP-1 or TRAPα, respectively. In C. elegans, transcription of hsp-4, which encodes a homolog of the human ER chaperone binding immunoglobulin protein, is induced by ER stress (38). While green fluorescence was not detectable in wild-type hsp-4::GFP reporter animals grown under normal culture conditions, it was readily visualized in trap-1 mutant animals harboring the same transgene (Fig. 4, E and F). Reduction of TRAPα activity in INS 832/13 cells did not induce IRE1-dependent splicing of xbp-1 mRNA (Fig. 4G) (38) but did result in increased phosphorylation of eukaryotic initiation factor 2α (eIF2α) at serine-51 (Fig. 4H), which can be phosphorylated...
by PERK in response to ER stress (39). Thus, we cannot exclude the possibility that TRAPα may indirectly influence distal events in insulin biogenesis through effects on ER stress that are not similarly consequential for AAT (Fig. 3, J and K).

**DISCUSSION**

Although the role of ER translocation in governing insulin biogenesis has been underappreciated, hints of its importance have emerged from studies on rare missense mutations in the signal peptide

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**Fig. 4. TRAPα promotes insulin biosynthesis and reduces ER stress.** (A) Immunostaining of INS832/13 wild-type or TRAPα KO cells with anti-TRAPα and (A) anti-insulin or (B) anti-proinsulin antibodies. Nuclei are stained with DAPI. (C) Immunostaining of TRAPα KO cells with anti-Myc and anti-insulin antibodies after transfection with a plasmid encoding a Myc-tagged TRAPα cDNA. Nuclei are stained with DAPI. (D) Quantification of insulin-positive cells in INS 832/13 wild-type, TRAPα KO, and TRAPα KO cells expressing exogenous Myc-tagged TRAPα. (E) Expression of the ER stress reporter hsp-4::GFP in second-stage (L2) and fourth-stage (L4) larvae and young adult wild-type (left) and trap-1(dp672) null mutant (right) animals. At each stage, images of wild-type and trap-1 mutant animals were captured with equivalent exposure times. Scale bars, 100 μm. (F) Higher-magnification images of representative adult wild-type (left) and trap-1 mutant (right) hsp-4::GFP transgenic animals. Images were captured with equivalent exposure times. Scale bars, 20 μm. (G) Detection of unspliced and spliced forms of xbp-1 mRNA in INS 832/13 cells after TRAPα knockdown (left) or KO (right). Tunicamycin-treated cells (TM) were used as a positive control. The band corresponding to spliced xbp-1 mRNA is denoted by the arrowheads. M, molecular weight marker; scr, scrambled siRNA control. (H) SDS-PAGE and anti–phospho-Ser51-eIF2 immunoblotting of lysates from INS 832/13 wild-type cells that were transfected with either scrambled (Sc) or TRAPα siRNA as indicated.
of human preproinsulin that are associated with congenital diabetes (40). The current work provides insight into the importance of the ER translocation machinery in insulin biogenesis by establishing preproinsulin as the first bona fide client protein for TRAP\(\alpha\). Our data are consistent with a model whereby TRAP\(\alpha\) directly promotes preproinsulin translocation and indirectly influences proinsulin maturation and insulin secretion, possibly through its effects on ER stress. Variation in TRAP\(\alpha\) expression and/or activity may be generally relevant to the pathogenesis of type 2 diabetes, as several common intronic single-nucleotide variants in the human Sstr1 gene (encoding TRAP\(\alpha\)) are associated with type 2 diabetes risk and pancreatic \(\beta\) cell dysfunction (41). Genes encoding TRAP subunits are among the most highly up-regulated genes in pancreatic \(\beta\) cells exposed to high glucose concentrations (42), and the transcriptional induction of TRAP\(\alpha\), TRAP\(\beta\), and TRAP\(\gamma\) in pancreatic \(\beta\) cells requires the unfolded protein response transducer IRE1\(\alpha\) (43). Thus, up-regulation of TRAPs in pancreatic \(\beta\) cells in the context of hyperglycemia may promote metabolic stability by both enhancing insulin biogenesis and attenuating ER stress. Understanding the mechanistic basis for TRAP\(\alpha\) action in insulin biogenesis may lead to new approaches to prevent and treat type 2 diabetes.

As mammalian TRAP\(\alpha\) is expressed widely (44), it surely must have biological functions that are distinct from its role in insulin biogenesis. These may include promoting the translocation of other proteins that enter the secretory pathway [such as prion protein (17)] and/or participating in ERAD (37). Mice homozygous for a C-terminal TRAP\(\alpha\)-ShBle-LacZ fusion allele die perinatally, and homozygous TRAP\(\alpha\)-ShBle-LacZ embryos are small and have cardiac outflow tract and endocardial cushion defects (44). Therefore, TRAP\(\alpha\) is likely involved in the translocation of other secreted proteins that play important roles in embryonic development. Defining specific features of translocated proteins that confer TRAP\(\alpha\) dependence (17, 45) will facilitate the identification of additional TRAP\(\alpha\) client proteins and the elucidation of other biological functions of TRAP\(\alpha\).

MATERIALS AND METHODS
Generation and maintenance of *C. elegans* strains

Strains were maintained on standard nematode growth media (NGM) plates seeded with *Escherichia coli* OP50. Double- and triple-mutant strains were constructed using conventional methods. *trap-1* mutant alleles were generated using CRISPR-Cas9–based genome editing (46). Two guide RNAs complementary to sequences in exon 2 were used. Molecular analysis of *trap-1* mutants is described in fig. S1. DNA encoding an mCherry epitope tag was inserted in-frame at the 3′ end of the *trap-1* open reading frame using CRISPR-Cas9–based homology-directed genome editing as described (47). Red fluorescent animals were isolated, and the mCherry insertion was verified by Sanger sequencing of polymerase chain reaction (PCR) products spanning the insertion site.

Genetic screen for modifiers of *C. elegans* DAF-2/InsR signaling

The suppressor of *eak-7;akt-1* (*seak*) screen was performed by mutagenizing *eak-7;akt-1* double mutants with N-ethyl-N-nitrosourea and screening for rare animals in the F\(_2\) generation that did not arrest as dauers as described. Genomic DNA isolated from *eak-7;akt-1* suppressor strains was sequenced and analyzed as described (5).

Dauer arrest assays

Dauer arrest assays were performed at 25°C as previously described (7). Crude dauer pheromone was isolated as described (12), and dauer pheromone assay was performed as described (4). Briefly, well-fed, gravid hermaphrodites were transferred to 35-mm assay plates containing 0, 2, or 10 \(\mu\)l of pheromone for a 2-hour synchronized egg lay at 20°C. Gravid animals were removed, and plates were transferred to 25°C and scored for dauers after an 80-hour incubation. For each experiment, 10 plates of animals of each genotype were scored for each of the three pheromone concentrations. The experiment was performed twice.

Scoring of eggs and viable progeny

Synchronized fourth-stage (L4) animals (at least eight animals per strain per experiment) were singly transferred onto 60-mm NGM agar plates seeded with half the normal volume of *E. coli* OP50 (50 \(\mu\)l) to improve visibility of eggs. Animals were incubated at 20°C, and eggs laid were counted manually every day using a dissecting microscope. Animals were transferred to new plates every 24 hours until the end of their reproductive life spans. Counts for each animal were summed to determine brood size. Serial, egg-laid plates were maintained at 20°C and evaluated for the number of viable larvae compared to the number of eggs identified within each plate, as an index of viability. Experiments were replicated three times by two independent evaluators.

Real-time quantitative PCR

Quantitative PCR was performed as previously described (48).

*C. elegans* fluorescence microscopy

To image TRAP-1::mCherry animals, well-fed animals were mounted on slides layered with a thin 3% agarose pad containing 25 mM sodium azide. Images were captured on a Leica inverted SP5 X inverted confocal microscope using Leica Application Suite Advanced Fluorescence software (Leica). For *hs-4p*::GFP reporter animals, wild-type and *trap-1* mutant animals harboring an integrated *hs-4p*::GFP transgene (38) were harvested, incubated in alkaline hypochlorite [500 mM NaOH and 1.2% (v/v) hypochlorite], and vortexed to isolate eggs. Eggs were washed twice with M9 buffer solution and incubated at 20°C on standard NGM plates seeded with *E. coli* OP50. Animals were imaged at L2 larval, L4 larval, and young adult stages on a Leica MZ16 F fluorescence stereomicroscope or an Olympus BX61 upright microscope. Three experimental replicates yielded similar results.

Reagents

Rat monoclonal anti-KDEL and rabbit monoclonal anti-GM130 antibodies were from Abcam (ab50601 and ab52649, respectively). Rabbit polyclonal anti-TRAP\(\alpha\) antibodies were from Novus Biologicals (NBP1–86912). A mouse monoclonal antibody that recognizes a human proinsulin C-peptide–A-chain junction peptide (GSLQKRGIVE) was raised by Abmart. Mouse monoclonal anti-tubulin antibodies were from Sigma (T5168). Guinea pig polyclonal anti-porcine insulin antibodies were from Millipore. Rabbit polyclonal anti-AAT antibodies were from Sigma (A001202-2). Rabbit polyclonal anti-HSP90 (no. 4874S), anti-eIF2 (no. 9722S), and anti–phospho-Ser51 eIF2 antibodies (no. 9721) were from Cell Signaling Technology. 35S-Met/Cys was from ICN. Dithiothreitol (DTT), protein A-agarose, digi-tonin, N-ethylmaleimide, and other chemical reagents were from...
Manipulating TRAPα expression in INS 832/13 cells

INS 832/13 cells lacking TRAPα were generated using CRISPR-Cas9–mediated genome editing (49). Single-guide RNAs were designed using guide design resources available on the Zhang Lab website (https://zlab.bio/guide-design-resources). Oligonucleotides corresponding to 5′-CACCGTCGACCTCGTGAAGCT-3′ 20-nucleotide guide sequences were annealed and ligated into the pSpCas9(BB)-2A-Puro (PX462) v2.0 vector as described (49). This plasmid was transfected into INS 832/13 cells using Lipofectamine 2000 (Thermo Fisher Scientific). Forty-eight hours after transfection, cells were plated and cultured with RPMI 1640 growth medium containing puromycin (1 µg/mL). Puromycin-resistant clones were scored for loss of TRAPα (“TRAPα KO”) by immunoblotting with anti-TRAPα antibodies. To reexpress TRAPα, TRAPα KO cells were transiently transfected with a plasmid encoding Myc-tagged human TRAPα. Forty-eight hours after transfection, insulin content was assayed by immunofluorescence.

Cell culture

Cells were plated into six-well plates 1 day before transfection. A total of 2 µg of plasmid DNA was transfected using Lipofectamine 2000 (Invitrogen). For metabolic labeling, cells were pulse-labeled with [35S-Met/Cys for 15 min, washed, and chased with label-free media for 0, 60, or 120 min as indicated. Immediately thereafter, media and cell lysate were immunoprecipitated with anti-insulin or anti-AAT antibodies, and immunoprecipitates were subjected to GFP or fluorescence SDS-PAGE under reducing conditions. Preproinsulin, proinsulin, insulin, and AAT were analyzed using a Typhoon Phosphorimager (GE Healthcare). Band intensities were quantified using National Institutes of Health ImageJ.

Immunofluorescence

Cells were permeabilized with 0.5% Triton X-100, fixed with formaldehyde, blocked, and incubated with anti-TRAPα, anti-KDEL, anti-GM130, anti-insulin, anti-proinsulin, or anti-Myc antibodies. After incubation with fluorophore-conjugated secondary antibodies, specimens were imaged with an Olympus FV500 confocal microscope.

Immunoblotting

Cell lysates were boiled in sample buffer containing 100 mM DTT for 5 min, resolved by 4 to 12% gradient SDS-PAGE, and transferred to nitrocellulose. Primary antibodies were used at 1:1000 dilution in tris-buffered saline with Tween 20 (0.1% Tween 20) plus 5% bovine serum albumin, with the exception of anti-HSP90, which was used at 1:2000 dilution. Secondary antibodies [goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP), goat anti-mouse IgG HRP (both from Bio-Rad), and goat anti-guinea pig IgG HRP (Jackson ImmunoResearch)] were used at 1:5000 dilution. Imaging was performed after incubation with the Clarity Western ECL Substrate (Bio-Rad no. 1705061) according to the manufacturer’s instructions.

Coimmunoprecipitation assay

The cross-linker dithiobis(succinimidyl propionate) (DSP; Thermo Fisher Scientific no. 22586) was freshly prepared at 20 mM in dimethyl sulfoxide and diluted to a working concentration of 0.5 mM in PBS. Cells were washed twice with PBS and incubated in 0.5 mM DSP for 30 min at room temperature. After removal of DSP, cells were incubated with quenching solution [20 mM tris-Cl (pH 7.4)] for 15 min at room temperature, lysed, and incubated with anti-TRAPα antibodies for 6 hours at 4°C. Immunoprecipitates were washed, subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-TRAPα and anti-proinsulin antibodies.

Glucose-stimulated insulin secretion assay

Glucose-stimulated insulin secretion assay was performed as previously described, with minor modifications (50). Briefly, INS 832/13 cells were washed and incubated with prewarmed Krebs-Ringer bicarbonate Hepes (KRHB; 0.5% bovine serum albumin, 129 mM NaCl, 5 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, and 10 mM Hepes (pH 7.4)) at 37°C for 2 hours. The supernatants were collected, and the cells were stimulated with KRHB containing either 2.8 or 25.5 mM glucose for 2 hours. After supernatants were collected, cells were lysed with acid ethanol, and insulin levels in supernatants and cell lysates were measured using STELLUX Chemi Human Total Proinsulin ELISA kits (ALPCO no. 80-PINHUT-CH01).

Statistical analyses

Statistical analyses were carried out by Student’s t test or one-way analysis of variance (ANOVA) using GraphPad Prism 7.

xbp-1 splicing assay

INS 832/13 cells were harvested after transfection with siRNAs or treatment with tunicamycin (final concentration of 2.5 µg/mL), total RNA was isolated using PureLink RNA Mini Kits (Invitrogen no. 12130318A), and cDNA was synthesized using First Strand cDNA Synthesis Kits (OriGene no. NP100042). The following primers were used to amplify spliced and unspliced xbp-1 transcripts: 5′-ATTCTTGACGGCTTGGCTCCT-3′ (forward) and 5′-CTCTGGGGAAGGGACATTGGA-3′ (reverse).

PCR products were subjected to 2.5% agarose gel electrophoresis. The size difference between products amplified from spliced and unspliced xbp-1 cDNA templates is 26 nucleotides.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/12/eaax0292/DC1

Fig. S1. trap-1 mutant DNA sequences and predicted effects on TRAP-1 protein.

Fig. S2. trap-1 and Y71F9AL.1 mutant phenotypes.
Table S1. Description of eight in TRAP α C. elegans TRAP-1 and human TRAP

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