Supplementary Material

Genome-wide screen identifies a novel p97/CDC-48-dependent pathway regulating ER stress-induced gene transcription

Esther Marza[1,2,4], Saïd Taouji[1,2*], Kim Barroso[1,2*], Anne-Aurélie Raymond[2,3*], Léo Guignard[2,4], Marc Bonneu[2,5], Nestor Pallares[1,2], Jean-William Dupuy[2,5], Martin E. Fernandez-Zapico[6], Jean Rosenbaum[2,3], Francesca Palladino[7], Denis Dupuy[2,4] and Eric Chevet[1,2,8,9]

[1]INSERM, UMR1053, Team «Endoplasmic Reticulum stress and cancer » F-33076 Bordeaux, France. [2]Univ. Bordeaux, F-33000, Bordeaux, France. [3]INSERM, UMR1053, « REPTeam » F-33076 Bordeaux, France. [4]INSERM U869, ARNA laboratory, F-33000 Bordeaux, France. [5]Plateforme Proteome, F-33000, Bordeaux, France. [6]Schulze Center for Novel Therapeutics, Division of Oncology Research, Mayo Clinic, Rochester, MN, USA. [7]Laboratory of Molecular and Cellular Biology, CNRS UMR5239, Université de Lyon, Ecole Normale Supérieure, 69364 Lyon Cedex 07, France. [8]Centre Régional de Lutte Contre le Cancer Eugène Marquis, 35000 Rennes, France.

*equally contributed to this work

[9]Correspondance to EC - Email: eric.chevet@inserm.fr

Supplementary Materials and Methods

C. elegans strains **-** Worms were grown at 20°C under standard conditions [1]. The N2 Bristol strain was used as wild-type. *cdc-48.1(tm544)* and *cdc-48.2(tm659)* strains were provided by Dr. S. Mitani (Tokyo Women's Medical University). The transgenic strain BC14636 (*dpy-5(e907); sIs13872[ckb-2p::gfp, pCeh361]*) was obtained from the British Columbia *C. elegans* Gene Expression Consortium and is described elsewhere [2]. The strains EM1 (*dpy-5(e907); cdc-48.1(tm544); sIs13872*) and EM2 (*dpy-5(e907); cdc-48.2(tm659); sIs13872)* were generated by crossing and the presence of the mutation verified using PCR.

Human cell lines, transfection and antibodies - Human HuH7 cells were cultured in complete Dulbecco's modified Eagles's medium containing 10% fetal bovine serum and antibiotics. HuH7 cells constitutively expressing a FLAG tagged reptin and HuH7 cells expressing a doxicyclin-inducible small hairpin RNA against *Reptin* were described previously [3]. Mouse antibodies recognizing Reptin or Pontin and rabbit antibodies recognizing Reptin were purchased from Abcam (Cambridge, MA, USA), and mouse antibodies recognizing the FLAG tag (M2 clone) were purchased from Sigma (StLouis, MO, USA). Anti Ubiquitin antibodies were from Santa Cruz Biotechnologies (SantaCruz, CA, USA). Anti-XBP1s were previously described [4]. ERSE::Tomato construct was kindly provided by E Snapp (Albert Einstein College of Medicine, NY, USA). Immunoprecipitations and Western blot analysis were performed as described previously [3, 5, 6]. DBeQ was kindly provided by Dr F Schoenen (MLPCN, KS, USA).

ER stress induction - For experiments carried out on solid medium, tunicamycin (TM, Calbiochem (EMD Biosciences Inc., Darmstadt, Germany) was added to NGM at a final concentration of 5 µg/ml before pouring plates. L4 synchronized worms were transferred to TM plates for 16 hours. For experiments in liquid culture, animals were exposed to media containing 0.5 µg/ml TM for 16 hours, unless specified otherwise. Dimethyl sulfoxide (DMSO) was used as control. Mammalian cells were treated with tunicamycin (5 µg/ml) or thapsigargin (0.5 nM) for various amounts of time as previously described [6, 7].

Sample preparation for mass spectrometry - BC14636 and EM2 synchronized young adult worms (48h after L1) were grown on OP50-seeded NGM plates and exposed to tunicamycin (5 ⌈g/ml) for 16h before being collected with S-basal, treated 1h with NaCl 0.1M at 20°C and frozen. Three independent pools for each condition have been used. Worms were thawed, resuspended into the lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 1M Tris-HCl pH8.5, phosphatase inhibitors (PhosphoStop), complete protease inhibitor (Roche)), sonicated twice

(15 sec power 5) and centrifuged at 3000 rpm for 10 min at 4°C. Proteins (60 ⌠g) were reduced in Laemmli buffer containing 30mM DTT for 30 min at RT with shaking. Iodoacetamide (90 mM) was added and samples were left another 30 min at RT, in the dark, with shaking. Proteins were separated on a 10% SDS gel and visualized by Coomassie Blue staining. Each gel lane was cut into 18 homogenous slices, destained in 25 mM ammonium bicarbonate, 50% Acetonitrile (ACN) and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature. Proteins were digested by incubating each gel slice with 10 ng/µl of trypsin (T6567, Sigma-Aldrich), 40 mM ammonium bicarbonate, 10% ACN overnight at 37°C. The resulting peptides were extracted from the gel in three steps: an incubation in 40 mM ammonium bicarbonate, 10% ACN for 15 min at room temperature and two incubations in 47.5 % ACN, 5% formic acid for 15 min at room temperature. The three collected extractions were pooled with the initial digestion supernatant, dried in a SpeedVac, and resuspended with 40 µl of 5% ACN, 0.1% formic acid before nanoLC-MS/MS analysis.

Mass spectrometry analyses - Online nanoLC-MS/MS analyses were performed using an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) coupled to a nanospray LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). Peptide extracts (10 µl) were loaded on a 300µm ID x 5 mm PepMap100 C18 µcolumn (Thermo Scientific) at a flow rate (30 µl/min) for 5 min and separated (200 nl/min, 115 min) on a 75 µm ID x 15 cm acclaim® C_{18}PepMap100 analytical column (Thermo Scientific) with a 5-40% linear gradient of 0.1% formic acid in 80% ACN. Capillary and needle voltages were 48-V and 1.8-kV, respectively. Data were acquired in a data-dependent mode alternating an FTMS scan survey over the range m/z 300–1700 with the resolution set to a value of 60 000 at m/z 400 and six ion trap MS/MS scans with Collision Induced Dissociation (CID) as activation mode. MS/MS spectra were acquired using a 3-m/z units ion isolation window and normalized collision energy of 35. Mono-charged ions and unassigned charge-state ions were rejected from fragmentation. Dynamic exclusion duration was set to 30s. The Mascot Daemon software (version 2.2.0, Matrix Science, London, UK) was used for protein identification in batch mode with the non-redundant *Caenorhabditis elegans* SwissProt database as a reference. Green fluorescent protein from the *Aequorea victoria* SwissProt database was added to the database. Two missed enzyme cleavages were allowed. Mass tolerances in MS and MS/MS were set to 10 ppm and 0.8 Da. Oxidation of methionine and carbamidomethylation on cysteine were searched as variable modifications. Mascot results were parsed with MFPaQ version 4.0.0 beta 5 software (Mascot File Parsing and Quantification, Toulouse, France), and protein hits were automatically validated with 1% protein FDR (evaluated using the “decoy”

option of Mascot). Only top ranking peptides with a Mascot score higher than the identify threshold at p = 0.05 were selected for quantification. Quantification of peptides was performed using the MFPaQ v4.0.0 beta software label-free module by XIC extraction and comparison of area under the curve (AUC) values of peptides with the same sequence, the same modification(s) and the same charge state. Area values were first normalized on the total AUC of all identified peptides. For each condition (EM2 or BC14636), only peptides with a coefficient of variation of area values ≤30% were kept. Further, only proteins supported by at least two peptides were included. Protein ratios were defined as the average of the ratios of the peptides assigned to the protein. Proteins were considered as variants if ≥70% of their peptides ratios were ≥2 or ≤0.5 and if the other peptides ratios were ≥1.4 and ≤0.71.

RNAi treatment – For low throughput experiments, L1 synchronized larvae were fed with RNAi-expressing bacteria grown on NGM plates containing 1mM IPTG and 50 µg/ml ampicillin, or in liquid culture using S-Medium plus 1mM IPTG and 50 µg/ml ampicillin for 2 days before being exposed to tunicamycin. The L4440 empty vector and *gfp* RNAi were used as controls.

Protein half-life determination – Reptin's half-life was determined using either i) cycloheximide treatment (100µg/ml final concentration) for 8h then treatment up to 120 min with tunicamycin or DBeQ or both as described elsewhere in the manuscript. Protein extracts were then resolved by SDS-PAGE and immunoblotted with anti Reptin antibodies or ii) ^{35}S-methionine (0.5 mCi/3ml medium met/cys free) pulse-labeling for 15 min following incubation of the cells for 1h with DMEM –Met/Cys, then chase in the presence of DMEM, 10% FBS in the presence or not of tunicamycin, DBeQ or both for up to 120 min (same conditions as in i)). Cells were then lysed using 100 µl of 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% SDS and Complete - protease inhibitors cocktail, then the lysate was diluted to 1 ml with 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TX-100. Lysate was spun down and immunoprecipitated for 3h on ice using rabbit anti Reptin antibodies prior adding protein A magnetic beads for 30 min. The immunoprecipitates were then washed extensively with 30 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TX-100, scintillation reagent added and directly counted on a scintillation counter.

Statistical analysis - Data are presented as mean ± SEM of at least three separate experiments if not specified otherwise and compared with multiple t-test corrected using the Holm-Sidak method for C. elegans experiments or the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests for experiments on mammalian cells. The significance

was set at P value < 0.05. All statistical analyses were performed using GraphPad Prism (version 6) statistical software (GraphPad Software; San Diego, CA).

Fluorescence microscopy - Live worms were immobilized in 10 mM sodium azide and observed on a Zeiss Axio Imager Z1 microscope, using a 10x objective. Images were treated using ImageJ [8].

Quantitative RT-PCR - Total RNA extraction and cDNA reverse transcription were performed as previously described [9].

Data representation – RNAi screen data and proteomics data were analyzed using the Panther software suite [10, 11] and the String program [12-14].

Data availability **-** The RNAi experiments have been given the following range of WormBase IDs: WBRNAi00094844 - WBRNAi00095088. The mass spectrometry data were deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000636 and DOI 10.6019/PXD000636.

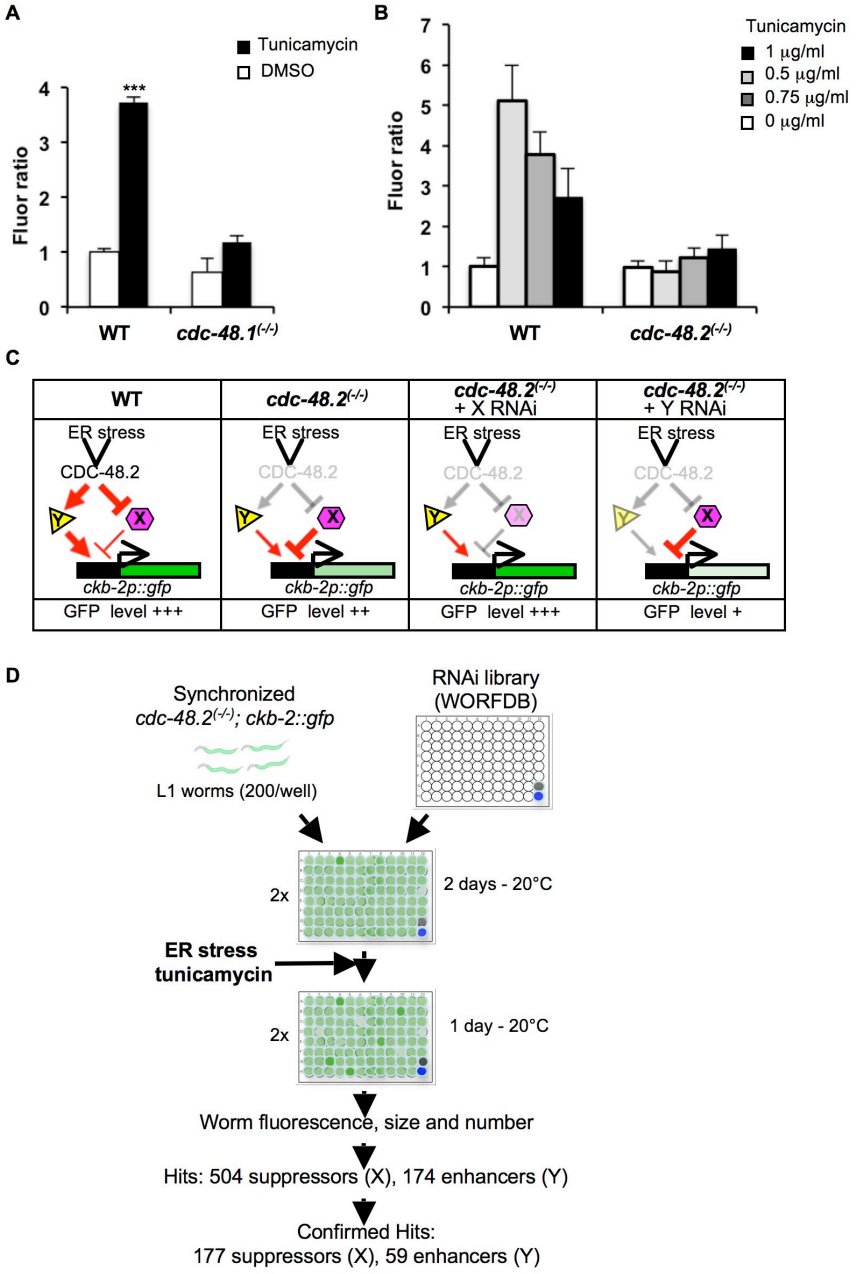

Figure S1. Genome-wide screen strategy.

(A) *cdc-48.1* was required to activate *ckb-2p::gfp* transcription in response to tunicamycin. Fluorescence intensities were quantified using flow cytometry. *ckb-2p::gfp* and *cdc-48.1*$^{(-/-)}$*; ckb-2p::gfp* worms were exposed to tunicamycin (0.5 µg/ml) or DMSO for 16 hours in liquid culture. F0 was defined as the fluorescence intensity obtained in *ckb-2p::gfp* worms fed with the empty vector and treated with DMSO. (Mean ± s.e.m, N = 8 with 200 worms per experiment). *P* value was calculated using multiple t-test corrected using the Holm-Sidak method **p < 0.001 ; *p < 0.01 **(B)** *cdc-48.2* was required to activate *ckb-2p::gfp*

transcription in response to tunicamycin. Fluorescence intensities were quantified using flow cytometry. *ckb-2p::gfp* and *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* worms were exposed to increasing concentrations of tunicamycin (0 to 1 µg/ml) for 16 hours in liquid culture. F0 was defined as the fluorescence intensity obtained in *ckb-2p::gfp* worms fed with the empty vector and treated with DMSO. (Mean ± s.e.m, N = 8 with 200 worms per experiment). *P* value was calculated using multiple t-test corrected using the Holm-Sidak method **p < 0.001 ; *p < 0.01. **(C)** Working model. CDC-48.2 could either activate an activator (Y) or inactivate a repressor (X) of *ckb-2* transcription specifically in response to ER stress. In *cdc-48.2*$^{(-/-)}$ mutants, *ckb-2* transcriptional activation upon ER stress is abolished. Down-regulating this activator or repressor by RNAi should respectively attenuate or restore *ckb-2::gfp* transcription under ER stress. (**D**) RNAi screening strategy. *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* synchronized L1 larvae were fed with the ORFeome WORFDB library in liquid culture in 96-well plates containing a positive control (blue) and a negative control (grey). Each RNAi plate was tested in duplicate. ER stress was induced by tunicamycin (0.5 µg/ml) for 16 hours. Average size, number and green fluorescence were measured using the COPAS biosort flow cytometer. A first set of suppressors (504) and enhancers (174) were selected and retested the same way (5 independent experiments), allowing the selection of confirmed suppressors (177) and enhancers (59).

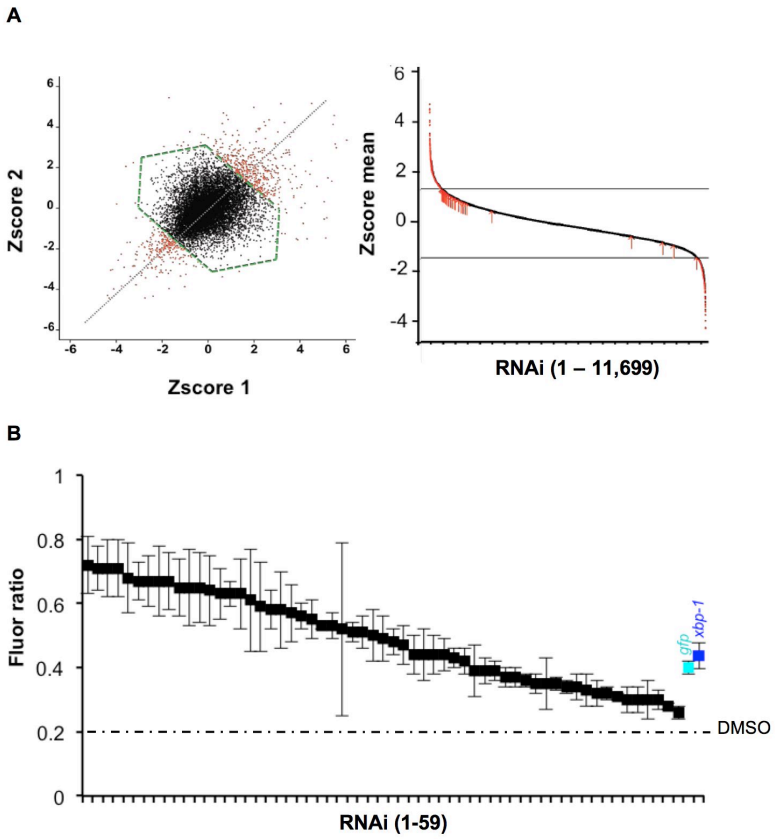

Figure S2. RNAi screen identifies *cdc-48.2*$^{(-/-)}$ mutation suppressors and enhancers on *ckb-2* expression in response to tunicamycin. (A) Left: Graph representing Zscore 1 in function of Zscore 2 calculated for each of the 11699 RNAi of the ORFeome library [15], which were performed in duplicates. (Pearson correlation coefficient: -0.00017). *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* synchronized L1 larvae were fed with RNAi in liquid culture up to L4 stage before being exposed to tunicamycin (0.5 µg/ml) for 16 hours. The fluorescence levels were quantified by flow cytometry. RNAi selected as suppressors or enhancers are shown in red. Right: Graph representing the Zscore mean obtained for each RNAi. The RNAi shown in red were selected for a second round of screening. **(B)** Fluorescence ratios of *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* worms fed with the enhancer RNAi (F). F0 represents the fluorescence intensity obtained with the empty vector. *gfp* RNAi was used as a control, and *xbp-1* RNAi was identified in the genetic RNAi screen. The dashed line indicates the value obtained when *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* worms were fed with the empty vector and treated with DMSO. (Mean ± s.e.m, N = 5 times 200 worms).

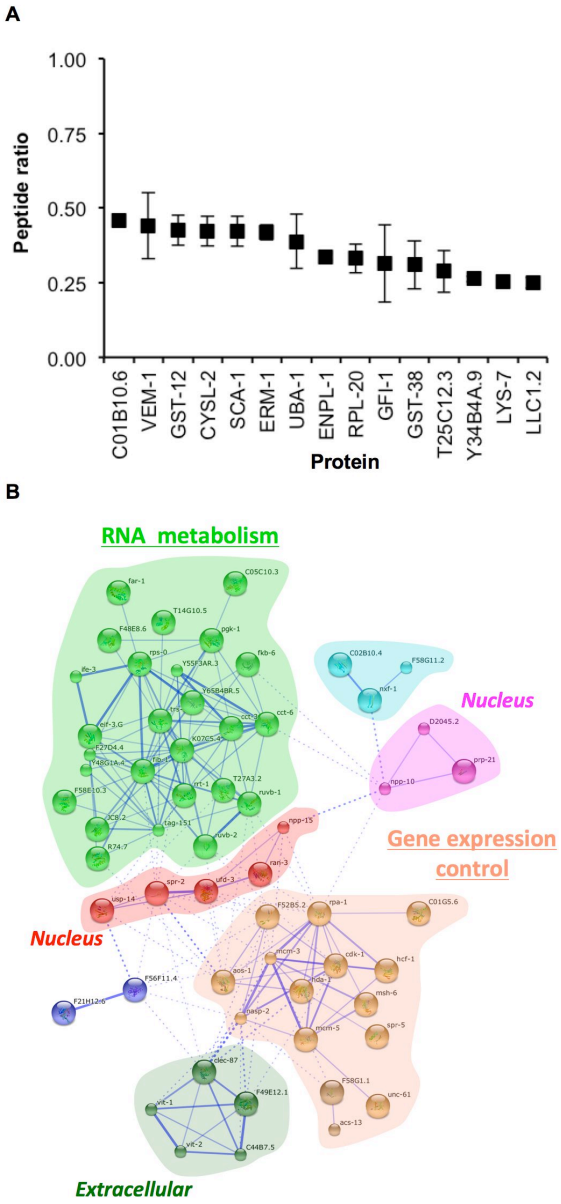

Figure S3. Identification of proteins that decrease in *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* mutants exposed to ER stress by mass spectrometry. Peptide quantity ratio ((*cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp)/(ckb-2p::gfp))* for the 15 proteins that were found to be less abundant in *cdc-48.2*$^{(-/-)}$ mutant background than in WT. *cdc-48.2*$^{(-/-)}$*; ckb-2p::gfp* and *ckb-2p::gfp* synchronized L1 larvae were grown to the L4 stage and exposed to tunicamycin (5 µg/ml) for 16 hours on NGM agar plates. Proteins were extracted and peptides were then identified and quantified by label-free LC-MS/MS mass spectrometry, as described in Fig 3. (Mean ± s.e.m, three independent experiments were performed). **(B)** The network was built using the String program suite and the advanced properties. Network connectivity was of 64% and 238 interactions were observed (77 expected, P val = 0).

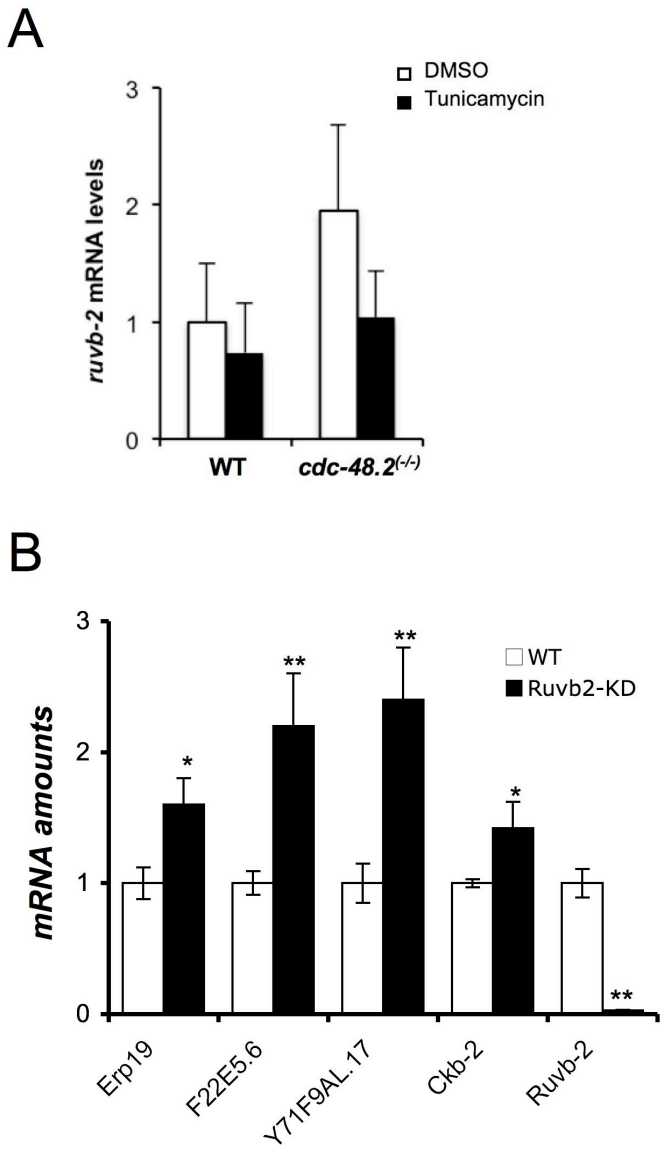

Figure S4. UPR^{ER} gene and *Ruvb-2* mRNA levels in *cdc-48.2$^{(-/-)}$; ckb-2p::*gfp and *ckb-2p::gfp* worms exposed or not to tunicamycin. (A) *Ruvb-2* mRNA levels were quantified by quantitative PCR on *cdc-48.2$^{(-/-)}$; ckb-2p::*gfp and *ckb-2p::gfp* adult worms exposed to tunicamycin (5 µg/ml) on NGM agar plates for 16 hours. *ruvb-2* mRNA levels were standardized by using the *ama-1* mRNA levels as an internal control for each condition. Values are normalized to the levels found in the WT background in presence of DMSO. More than 1000 worms were used to extract the RNA in each condition. Total RNA extraction, reverse transcription and qPCR protocol are described elsewhere [9] (Mean ± SD, N = 3). (B) RT-qPCR quantification of the relative expression levels of 3 endogenous ER homeostasis genes (ERp19, F22E5.6, Y71F9AL.17/COPA-1), Ckb-2 and Ruvb-2 under basal conditions in *WT* worms subjected or not to *ruvb-2* RNAi. Bars represent the mean of 3 biological replicates. (Mean ± s.e.m, N=3) ***P*<0.01; ****P*<0.001

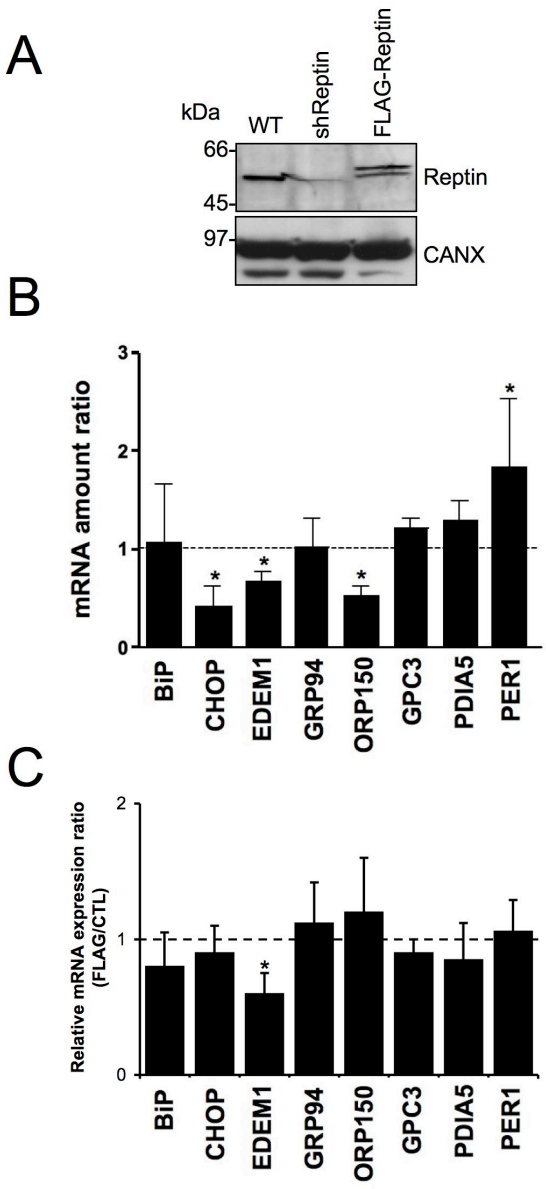

Figure S5. Impact of ectopic expression of FLAG reptin on UPR^{ER} gene expression. (A) Western blot analysis of retpin expression in control cells, in cells silenced for reptin and in cells expressing FLAG reptin. **(B)** Ratio of expression of UPR^{ER} genes in HuH7 control and HuH7-FlagReptin cells under basal conditions as determined by RT-qPCR (HuH7-FlagReptin/HuH7 control). The experiment was carried out three times on biological triplicates. **P*<0.03. **(C)** Ratio of expression of UPR^{ER} genes in HuH7 control and HuH7-FlagReptin cells upon tunicamycin treatment (2 ⌈g/ml for 8h) as determined by RT-qPCR.The experiment was carried out three times on biological triplicates. **P*<0.03.

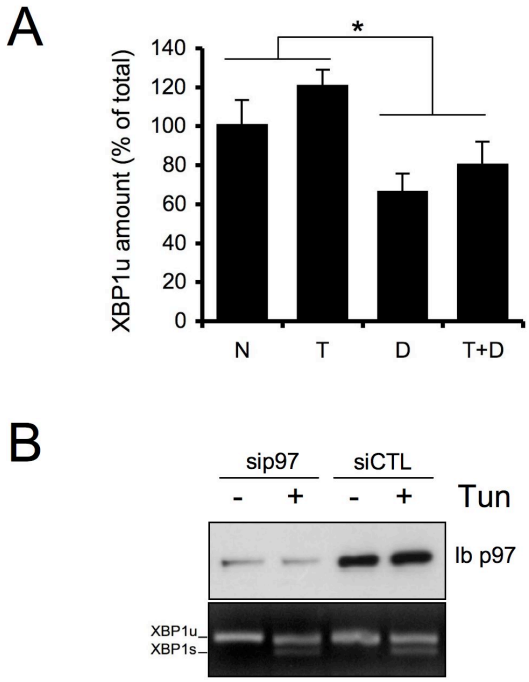

Figure S6. Impact of the modulation of p97/CDC-48 activity/expression on XBP1 mRNA. (A) HuH7 cells were treated either with DBeQ (20 µM, D), tunicamycin (2 ⌠g/ml; T) or both for 4 hours. Unspliced XBP-1 mRNA levels were determined by RT-PCR (Mean ± SD, N = 3). *P* values were calculated using multiple t-test corrected using the Holm-Sidak method. **P* < 0.05**. (B)** HuH7 cells were transfected with a siRNA to p97/CDC-48 or a siRNA control and 48h after transfection treated either tunicamycin (2 ⌠g/ml; T) for 4 hours. XBP-1 mRNA splicing was determined by RT-PCR.

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