Supplemental Information

SI Materials and Methods

Cloning, Expression, and Purification

All constructs were derived from human cDNA specifically the major Bag6 isoform 2. For crystallization, both Bag6 (1000 to 1054) and TRC35 (23 to 305) were inserted into the multiple cloning site of the pACYCDuet plasmid (Novagen) with an N-terminal hexahistidine tag on Bag6(1000-1054). The plasmid was transformed into *E. coli* NiCo21(DE3) (New England Biolabs). The plate was scraped to inoculate 12 2L baffled flasks containing 2xYT media then grown by shaking at 37°C in a shaking incubator (Multitron Standard Infors HT) at 250 rpm. Cell growth was monitored to an OD_{600} = 0.6 then protein expression was induced for 3 hours by the addition of 500 µM isopropyl-β-D-thiogalactopyranoside (IPTG) (Affymetrix). Cells were harvested by centrifugation in a TLA 8.1 rotor at 4000 ×g for 20 minutes. The pellet was resuspended in 50 mM MOPS pH 7.2, 300 mM K•glutamate, 5 mM MgOAc, 20 mM imidazole, 5 mM β-mercaptoethanol (1g cell / 10 mL lysis buffer) and supplemented with 0.1 mM PMSF and 1 mM benzamidine. Cells were lysed using an M-110L microfluidizer (Microfluidics) by two passes at approximately 17,500 psi. The lysate was clarified by centrifugation at 235,000 ×g in a Beckman Ti45 rotor for 30 minutes at 4°C. The clarified lysate was incubated for 1 hour with 3 mL of a 50% (vol/vol) slurry of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) by rocking. The mix was poured into a gravity column then washed with 100 mL lysis buffer. The protein was eluted with 12 mL elution buffer (20 mM MOPS (pH 7.2), 150 mM K•glutamate, 300 mM Imidazole, 5 mM β-mercaptoethanol). The eluent was mixed with ~0.5 mg of TEV protease in snakeskin dialysis tubing with 10 kDa molecular weight

cutoff (Thermo Fisher Scientific) and dialyzed overnight at room temperature in (20 mM Mops (pH 7.2), 100 mM K•glutamate, and 10 mM β-mercaptoethanol). Precipitate was removed by centrifugation with Beckman SX4750A rotor at 3000 ×g for 5 minutes at 4 °C and filtered with a 0.22 µm syringe filter. The sample was concentrated and loaded onto a 5 mL UnoQ ion-exchange column (Biorad) then eluted with a 60 mL 20 mM MOPS (pH 7.2), 50 - 500 mM K•glutamate gradient, 5 mM β-mercaptoethanol. The fractions containing the protein (~5-23mL) were dialyzed in snakeskin dialysis bag in 20 mM Mops (pH 7.2), 50 mM K•glutamate, 5 mM β-mercaptoethanol for 2 hours at 4 °C. The sample was concentrated to 2 mL, filtered with a 0.22 µm syringe filter, then further purified by size-exclusion chromatography over a 120 mL Superdex 75 column (GE Healthcare) (20 mM MOPS (pH 7.2), 50 mM K•glutamate, 5 mM β-mercaptoethanol). The fractions containing the heterodimer (61 mL – 72 mL) and monomeric Bag6 (77 mL – 82 mL) were pooled and concentrated to ~10 mg/mL using centrifugal filter units with 10 kDa molecular weight cutoff (Merck Millipore).

The plasmid containing human KPNA2 was obtained from Addgene (#26677). A truncated KPNA2 (58-529) was subcloned into pMAL-C2 vector (New England Biolabs) and transformed into *E. coli* NiCo21(DE3). The cells were grown in 2×YT media and induced at 37 °C until OD_{600} = 0.1 then cooled on ice for 1 hour. Expression was induced with 500 µM IPTG for 18 hours at 16°C in a shaking incubator at 200 rpm. The cells were harvested as above and resuspended in 50 mM MOPS (pH 7.2), 300 mM K•glutamate, 5 mM β-mercaptoethanol, supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche) and lysed using the M-110L microfluidizer by two passes at approximately 17,500 psi. The lysate was clarified by centrifugation at 235,000 ×g in a

Beckman Ti45 rotor for 30 minutes at 4°C. The lysate was incubated for 1 hour with 3 mL of a 50% (vol/vol) slurry of amylose resin (New England Biolabs) by rocking. The mix was poured into a gravity column then washed with 100 mL lysis buffer. The protein was eluted with 12 mL elution buffer (20 mM Mops (pH 7.2), 150 mM K•glutamate, 10 mM maltose, 5 mM β-mercaptoethanol). The sample was placed in snakeskin dialysis bag (10 kDa cutoff, Thermo Fisher) and dialyzed overnight at 4 °C in 20 mM Mops (pH 7.2), 50 mM - 800 mM K•glutamate gradient, 5 mM β-mercaptoethanol. The fractions containing the protein (~36 - 70 mL) were pooled and dialyzed in snakeskin dialysis bag in 20 mM Mops (pH 7.2), 50 mM K•glutamate, 5 mM β-mercaptoethanol for 2 hours at 4 °C. The sample was concentrated to 2 mL, filtered with a 0.22 µm syringe filter, and purified by size-exclusion chromatography over a 120 mL Superdex 200 column (GE Healthcare) (20 mM MOPS (pH 7.2), 50 mM K•glutamate, 5 mM β-mercaptoethanol). Fractions containing the sample (60 mL – 90 mL) were pooled and concentrated with centrifugal filtration units with 50 kDa molecular weight cutoff.

For expression and purification of GST•TRC35, full-length TRC35 was subcloned into pGEX6P-1 (GE Healthcare) and transformed into NiCo21(DE3). The cells were grown at 37 °C until OD_{600} = 0.1, chilled on ice for 1 hour, then induced with 500 µM IPTG at 16 °C for 18 hours in a shaking incubator at 200 rpm. Cells were lysed using 10 mL per g cell pellet lysis buffer (50 mM Hepes (pH 7.2), 400 mM KCl, 5 mM mercaptoethanol) and incubated with 3 mL 50% (vol/vol) slurry of glutathione resin (GE healthcare) for 2 hours at 4 °C by rocking. The resin was washed with 100 mL lysis buffer and the protein was eluted with 12 mL of freshly prepared 20 mM Hepes (pH 7.2), 150 mM KCl, 33 mM glutathione, 5 mM β-mercaptoethanol. The sample was placed in snakeskin dialysis bag

(10 kDa cutoff, Thermo Fisher) and dialyzed overnight at 4 °C in 20 mM Hepes (pH 7.2), 100 mM K•glutamate, 5 mM β-mercaptoethanol, before concentration with centrifugal filter unit (50 kDa molecular weight cutoff, Millipore).

Crystallization

Crystallization screening was performed using the sitting drop vapor-diffusion method with the commercially available PEG Ion screen (Hampton Research) and MRC 2 Well Crystallization plate (Hampton Research). Initial screening was performed by a Mosquito robot (TTP Labtech) with 100nL:100nL drops (protein:well solution) and 50uL wells. No refinement was necessary. Crystals grew to full-size after 5 days as rectangular prisms in 20% PEG-3350 (wt/vol), 0.2 M DL-malic acid (pH 7.0). For cryo-protection, crystals were transferred into 100uL of well solution with 20% (vol/vol) glycerol for 15 seconds and then frozen in liquid nitrogen. Rubidium derivatives were generated by transferring crystals into 40 µl well solution plus 350 mM rubidium iodide for 2 to 10 seconds prior to cryopreservation with glycerol.

Data Collection, Structure Solution, and Refinement

X-ray diffraction data were collected on beam line BL12-2 at the Stanford Synchrotron Radiation Laboratory (SSRL). A complete native dataset was collected from a single crystal to 1.8 Å resolution and a single rubidium derivative crystal dataset was collected from a single crystal to 2.0 Å with a Dectris Pilatus 6M detector. Data were integrated, scaled, and merged using XDS (1) and SCALA (2, 3). Phases were determined by molecular replacement single-wavelength anomalous dispersion (MRSAD) using SHARP

(4) and PHASER-MR (5) on PHENIX (6). The initial structure was built by PHASER as implemented by PHENIX (6). The model was further built and refined against the native dataset over several rounds using COOT (7) and PHENIX (6). Statistics are provided in Table 1.

Phylogenetic Tree of GET/TRC Components

The phylogenetic tree was modified from the maximum likelihood phylogenetic tree of eukaryotes by Eme *et al* (8). The MEME suite (9) was used to determine the presence or absence of the GET/TRC components in the genomes presented in the tree. The MEME motif discovery tool was used to make motifs for Ubl4A, Get5, Get4/TRC35, and Sgt2, SGTA, and Bag6. The motifs were then used to search the genomes using the motif scanning tool MAST (10). Identified proteins were confirmed using BLAST (11) and the corresponding reference ID is provided on table S2.

Yeast Two-Hybrid

The PJ69-4α strain was obtained from the Yeast Resource Center at the University of Washington. Bag6 isoform 2 residues 951 to 1126 were cloned into pGAD-C1 vector and TRC35 was cloned into pGBDU-C1 vector. Alanine mutations were made using Q5 site-directed mutagenesis (New England Biolabs). pGAD-C1-TRC35 (wt or mutant) and pGBDU-C1-Bag6 (wt or mutant) were co-transformed into PJ69-4α using previously described methods (12) then plated onto SC-Ura-Leu plates and incubated at 30 °C. Single colonies from the transformants were cultured in 5 mL SC-Ura-Leu liquid media and grown overnight in a shaking incubator (Multitron Standard Infors HT) at 200 rpm at

30 °C. 2 × 10^7 cells were transferred into total 5 mL SC-Ura-Leu media and grown in a shaking incubator at 30 °C at 200 rpm for 6 hours. Cells were harvested by centrifugation with a Beckman SX4750A rotor at 3000 ×g at 25 °C. The cells were washed twice by resuspension in 5 mL sterile water followed by centrifugation at 3000 ×g at 25 °C. After the second wash, cells were resuspended in 1 mL sterile water. Concentration was measured and 1 × 10^7 cells were resuspended in total 40 µL of sterile water. 4 µL of this resuspended sample were spot plated onto SC-Ura-Leu-Ade plate and incubated for 72 hours at 30 °C.

Yeast Two-hybrid Expression Controls

Cells were grown overnight in SC-Ura-Leu medium. 2.5 x 10^7 cells were transferred to a total 5 mL media and grown for ~6-8 hours until OD_{600} = 2.0. Cells were harvested by centrifugation at 4000 ×g for 5 minutes at 4 °C. Cells were resuspended in 2 mL 10 mM TE buffer (Tris-HCl, pH 8.0, 0.1 mM EDTA). Cells were centrifuged in a microcentrifuge at 13,500 ×g at 4 °C for 2 minutes. ~37.5 µL glass beads (415-600 µm size) for each OD unit were added to the sample. Same volume of 1x SDS-PAGE loading buffer was added. The cells were vortexed and immediately plunged into a boiling water bath. After boiling for 3 minutes, tubes were immersed into an ice bucket. The tubes were placed into FastPrep (MP biomedicals) homogenizer at 6 m/s for 45 seconds. Cells were boiled for 3 minutes and centrifuged in a microcentrifuge for 2 minutes at 13,500 g at room temperature. Supernatant was transferred to a clean microfuge tube and loaded onto 5-20% gradient gel (Biorad). The protein was transferred onto PVDF membrane. Membrane was blocked with Odyssey TBS blocking buffer (Li-Cor) for 1 hour. The membrane was

incubated with primary antibody against Gal4 DNA binding domain (Santa Cruz) for TRC35 detection or Gal4 trans-activating domain (Santa Cruz) for Bag6 detection. After washing with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20) for 10 minutes 3 times, membrane was incubated with anti-mouse secondary antibody (IRDye 680RD or 800CW, Li-Cor) for 1 hour at room temperature. Membrane was washed 3 times with TBST for 10 minutes each. Proteins were detected using an Odyssey Imaging System (Li-Cor).

Immunoprecipitation from 293T Cells

On day 0, 5 x 10^5 $Bag6^{-/-}$ cells were seeded in plates. On day 1, TRC35 with a C-terminal FLAG tag (TRC35•FLAG), Bag6 with a C-terminal GFP tag (wt or mutant Bag6•GFP), and HA•ubiquitin were co-transfected. On day 3, cells were collected and washed with 1 mL of ice cold phosphate-buffered saline. The cells were lysed with NP40 lysis buffer containing 1 mM N-ethylmaleimide to inhibit the ubiquitin-proteasome system. The detergent soluble fraction was used for immunoprecipitation (IP) using GFP antibody. After IP, the beads were divided into two fractions. Half was analyzed directly with SDS-PAGE and immunoblotting with indicated antibodies to assess the interaction between TRC35 and Bag6 and Bag6 mutants. The other half was used for denaturing immunoprecipitation to detect TRC35 ubiquitylation. The beads were resuspended in 150 µL denaturing buffer (1× PBS, 1% SDS, 5 mM DTT) and heated at 95 °C for 10 minutes. Then 1.35 mL NP40 lysis buffer was added into the tube and incubated at 4 °C for 30 minutes. The supernatant was used for immunoprecipitation with FLAG antibody

conjugated M2 beads (Sigma-Aldrich). The eluate was analyzed with SDS-PAGE and immunoblotting.

For RNF126 knockdown, 5×10^5 wild type 293T cells were seeded in 6 well plates. On day 1, cells were transfected with 60 pmol pooled RNF126 siRNAs (Thermo Fisher) or control siRNA using Lipofectamin RNAiMAX (Thermo Fisher). The sequence of the RNF126 siRNAs are GCAUCUUCGAUGACAGCUU (catalog number S31185), GAUUAUAUCUGUCCAAGAU (catalog number S31186), and GCAGGGCUACGGACAGUUU (catalog number S13387). Cells were passaged in 1:2 ratio onto new plates on day 2. Cells were transfected with TRC35•FLAG (wt), Bag6•GFP (wt or mut) and HA•ubiquitin on day 3. On day 4, the cells were collected and cell extracts were assayed with sequential immunoprecipitation and immunoblotting as above. To assay TRC35 ubiquitylation after proteasome inhibition, 5×10^5 wild type 293T cells were seeded in plates on day 0. On day 1, cells were transfected as above. 24 hour post transfection, proteasome inhibitor, MG132, was added to the concentration of 10 μM and incubated overnight. Cells were collected and analyzed with sequential immunoprecipitation and immunoblotting as above.

To investigate the effect of simultaneous RNF126 down-regulation and proteasomal inhibition on TRC35 ubiquitylation, 5×10^5 Bag6 293T cells were seeded in plates on day 0. On day 1, cells were transfected with RNF126 siRNA or control siRNA. On day 2, cells were split in half into new plates. After ~9 hours, plasmids encoding TRC35•FLAG (wt), Bag6•GFP (wt or mutant) and HA•ubiquitin were transfected. On day 3, medium was

replaced with fresh medium containing either 10 µM MG132 or DMSO. On day 4, cells were collected and sequential IP was performed as described above.

Localization Assay

On day 0, 1 x 10^5 Cos7 cells were seeded onto a 12-well plate with a poly-D-lysine coated cover glass. After approximately 8 hours, the cells were co-transfected with TRC35•FLAG and Bag6•GFP (wt or mutant). 20 hours after transfection, the cells were washed with 1× PBS, fixed with 4% (vol/vol) paraformaldehyde for 15 minutes, then washed with 1× PBS before permeabilization with staining solution (1× PBS, 5% fetal bovine serum (vol/vol), 0.1% (vol/vol) NP40). Cells were then stained with staining solution containing primary antibody and incubated at room temperature for 1 hour. After washing the cells with 1× PBS, the cells were incubated with staining solution with secondary antibody then washed with 1× PBS. Cells were counterstained with a mounting medium containing DAPI to illuminate the nucleus for visualization with Axiovert 200M microscope (Zeiss). Confocal microscope analyses were performed with a Zeiss LSM 780 system.

To assay the localization of each Bag6 mutant, 3 view fields were evaluated in 40X oil immersion objective. Approximately 100 cells were evaluated for each Bag6 variant and cells were counted as having Bag6 primarily in either the nucleus or in the cytosol.

***In vitro* Binding Assay with Purified Proteins**

1 nmol hexahistidine-tagged Ubl4A-Bag6C131 (wt, 1025SL or 1043SL) was incubated with 1 nmol GST•TRC35 or 1 nmol MBP•KPNA2 in 100 µL total volume of binding buffer

(20 mM MOPS (pH 7.2), 100 mM K•glutamate 20 mM imidazole, 5 mM β-mercaptoethanol) at room temperature for 30 minutes. 30 µL 50% slurry of Ni-NTA beads (Qiagen) equilibrated with binding buffer were added to the reaction and incubated at room temperature for 30 minutes. The beads were resuspended using a pipet every 10 minutes. The beads were then washed twice with 100 µL room temperature binding buffer. Samples bound to the resin were eluted with 25 µL elution buffer (20 mM Mops (pH 7.2), 100 mM K•glutamate, 300 mM imidazole, 5 mM β-mercaptoethanol) and evaluated with Coommassie-stained SDS-PAGE gel.

Exchange Assay

1 nmol hexahisidine-tagged Ubl4A-Bag6C131 (wt, 1024SL, or 1043SL) was incubated with 1 nmol GST•TRC35 in 100 uL total volume of binding buffer (20 mM Mops (pH 7.2), 100 mM K•glutamate, 20 mM imidazole, 5 mM β-mercaptoethanol). 30 µL 50% (vol/vol) slurry of glutathione beads (GE Healthcare) equilibrated with binding buffer were added to the reaction and incubated at room temperature for 30 minutes. The beads were resuspended every 10 minutes. The beads were washed twice with 100 µL room-temperature binding buffer (20 mM Mops (pH 7.2), 100 mM K•glutamate, 5 mM β-mercaptoethanol) then 0.5, 1 or 2 nmol MBP•KPNA in 100 µL was added to the resin and incubated for 30 minutes at room temperature. The beads were washed twice with 100 µL binding buffer then eluted with 25 µL elution buffer (20 mM Mops (pH 7.2), 100 mM K•glutamate, 33 mM glutathione, 5 mM β-mercaptoethanol). The reverse experiment starting with hexahistidine-tagged Ubl4A-Bag6C131 and MBP•KPNA2 was carried out as above but using 30 µL 50% (vol/vol) slurry of amylose beads (New England Biolabs) in

20 mM MOPS (pH 7.2), 100 mM K•glutamate, 5 mM β-mercaptoethanol and eluted with 25 µL of 20 mM MOPS (pH 7.2), 100 mM K•glutamate, 10 mM maltose, 5 mM β-mercaptoethanol.

Figure Legends

Figure S1. Comparison of TRC35 and Get4 structures. (a) Representative structures of fungal Get4 homologs. 3LKU is from *Saccharomyces cerevisiae* (*Sc*) and 3LPZ is from *Chaetomium thermophilum*. (b) Aligned human TRC35 (color ramped) and *Sc*Get4 (3LKU, grey) using Pymol (13) super for sequence-independent structural alignment. Left, structures aligned to the six N-terminal helices. Right, structures aligned to the seven C-terminal helices. (c) Accessible surface representation of TRC35 colored based on percent conservation as implemented in Chimera (14). Conservation based on a MAFFT (15) alignment of TRC35/Get4 sequences from *Homo sapiens*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, *Nematostella vectensis*, *Monosiga brevicollis*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Bag6 is in ribbons representation in pink. (d) The Bag6-binding surface of TRC35 colored based on hydrophobicity (Kyle-Doolittle scale) and percent conservation as implemented in Chimera (14).

Figure S2. Survey of factors involved in the TRC pathway in eukaryotes. A condensed phylogenetic tree of representative eukaryotes was built based on the maximum likelihood phylogenetic tree of eukaryotes by Eme *et al* (8). The genome of each organism was searched for the presence of Get4/TRC35, Get3/TRC40, and Bag6

using MEME suite motif discovery tool (MEME) (9) and motif scanning tool (MAST) (16) in addition to NCBI protein BLAST (11). Proteins are color coded and sequence elements are highlighted. Black circles indicate homologs that are missing residues demonstrated to be critical in fungal TA-targeting studies. Pink asterisk indicates that although a clear Bag6 homology could not be identified in the genome of *Caenorhabditis elegans*, Bag6-like proteins have been identified in genomes of other nematodes: *Brugia malayi*, *Loa loa*, *Wuchereria bancrofti*, and *Toxocara canis*.

Figure S3. Representative aligned sequences of eukaryotic TRC35/Get4. Species selected based on the eukaryotic phylogenetic tree by Eme *et al*. (8). Sequences were aligned with MAFFT (15). α-helices, based on the TRC35 structure, are highlighted in colors that correspond to the crystal structure on Figure 1B and numbered on top. Residues highlighted in red boxes were identified as critical to fungal Get4 binding Get3 (17). Residues highlighted in blue boxes are critical to Get4/TRC35 regulating Get3/TRC40 (17, 18). The residues that comprise the fungal β-hairpin are highlighted in a black box. The arrow indicates the end of the crystallization construct. Purple boxes highlight glutamate and aspartate residues in the C-terminus of TRC35.

Figure S4. Validation of the Bag6-TRC35 interface.

(A) Yeast 2-hybrid assay to validate the interface identified in the crystal structure. Wild-type or mutant full length TRC35 conjugated to the DNA binding domain was expressed with wild-type Bag6(951-1126) conjugated to the transcription activating domain. Transformation was confirmed by ability to grow on SC-Ura-Leu plates. Interaction was

determined by ability to grow on SC-Ura-Leu-Ade media. (B) Western blot analysis of yeast whole cell lysate for proper expression of prey and bait proteins. Whole cell lysates of PJ694α (without plasmid), PJ6 (PJ694α transformed with wtBag6), PJ-35 (PJ694α transformed with wtTRC35), PJ6+35 (PJ694α transformed with wt Bag6 and wtTRC35), PJ6(1004A/1036A) (PJ694α transformed with wtTRC35 and Bag6(W1004A/Y1036A), PJ6(1012A/1036A) (PJ694α transformed with wtTRC35 and Bag6(W1012A/Y1036A), and PJ35(Y262A) (PJ694α transformed with wtBag6 and TRC35(Y262A) were analyzed by immunoblotting with indicated antibodies. (C) Wild-type full-length TRC35 conjugated to the DNA binding domain was expressed with wild-type or mutant Bag6(951-1126) conjugated to the transcription activating domain. (D & E) Wild-type or mutant Bag6•GFP was co-expressed in Bag6$^{-/-}$ 293T cells with TRC35•FLAG and immunoprecipitated using anti-GFP antibody. Amount of TRC35 retrieved by Bag6 was assessed by blotting with anti-FLAG antibody. The positions of the higher molecular weight TRC35•FLAG is highlighted with asterisks.

Figure S5. Ubiquitylation of TRC35 in cells expressing Bag6 mutants.

(A) Immunoprecipitation of TRC35 in Bag6$^{-/-}$ 293T cells co-transfected with plasmids encoding TRC35•FLAG (wt), Bag6•GFP (wt or mutants), and HA•ubiquitin. Anti-GFP antibody was used for the first IP. Anti-FLAG antibody was used for the second IP in denaturing conditions. TRC35 ubiquitylation was assessed by immunoblotting with anti-HA antibody. The exposure times were adjusted to improve visibility of the reactive bands. (B) IP was carried out as in (A) in Bag6$^{-/-}$ 293T cells co-transfected with plasmids encoding TRC35•FLAG (wt) and Bag6•GFP (wt or mutants). TRC35 ubiquitylation was assessed

by immunoblotting with anti-FLAG antibody. The exposure times were adjusted to improve visibility of the reactive bands. (C) The cell extract used for immunoprecipitation was immunoblotted for Bag6, TRC35, and HA•ubiquitin with Bag6 antibody, FLAG antibody and HA antibody, respectively.

Figure S6. The effect of RNF126 and proteasome inhibition on TRC35 ubiquitylation. (A) Sequential denaturing immunoprecipitation analysis of the effect of RNF126 on TRC35 ubiquitylation. of wt293T cells co-transfected with plasmids encoding TRC35•FLAG (wt), Bag6•GFP (wt or mutants), and HA•ubiquitin. A combination of 3 siRNAs against RNF126 was used. Anti-GFP antibody was used for the first IP. Anti-FLAG antibody was used for the second immunoprecipitation in denaturing conditions. TRC35 ubiquitylation was assessed by immunoblotting with anti-HA antibody. (B) The ratio between ubiquitylated TRC35 and unmodified TRC35 from (A). The ratio of Ub-TRC35/TRC35 in the cell transfected with TRC35•FLAG, wtBag6•GFP and control siRNA is defined as 1. (C) Sequential immunoprecipitation was carried out as in (A) in 293T cells treated with MG132 and co-transfected with plasmids encoding TRC35•FLAG (wt), Bag6•GFP (wt or W1004A/Y136A), and HA•ubiquitin. TRC35 ubiquitylation was assessed by immunoblotting with anti-HA antibody.

1. Kabsch W (2010) Xds. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):125-132.
2. Collaborative Computational Project N (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr* 50(Pt 5):760-763.
3. Winn MD*, et al.* (2011) Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* 67(Pt 4):235-242.

4. Bricogne G, Vonrhein C, Flensburg C, Schiltz M, & Paciorek W (2003) Generation, representation and flow of phase information in structure determination: recent developments in and around SHARP 2.0. *Acta Crystallogr D Biol Crystallogr* 59(Pt 11):2023-2030.
5. McCoy AJ (2007) Solving structures of protein complexes by molecular replacement with Phaser. *Acta Crystallogr D Biol Crystallogr* 63(Pt 1):32-41.
6. Adams PD*, et al.* (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66(Pt 2):213-221.
7. Emsley P, Lohkamp B, Scott WG, & Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66(Pt 4):486-501.
8. Eme L, Sharpe SC, Brown MW, & Roger AJ (2014) On the age of eukaryotes: evaluating evidence from fossils and molecular clocks. *Cold Spring Harb Perspect Biol* 6(8).
9. Bailey TL*, et al.* (2009) MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res* 37(Web Server issue):W202-208.
10. Bailey TL & Gribskov M (1998) Combining evidence using p-values: application to sequence homology searches. *Bioinformatics* 14(1):48-54.
11. Altschul SF, Gish W, Miller W, Myers EW, & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403-410.
12. Gietz RD & Schiestl RH (2007) Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature protocols* 2(1):38-41.
13. Schrodinger, LLC (2015) The PyMOL Molecular Graphics System, Version 1.8.
14. Pettersen EF*, et al.* (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* 25(13):1605-1612.
15. Katoh K & Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30(4):772-780.
16. Bailey TL & Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol* 2:28-36.
17. Gristick HB*, et al.* (2014) Crystal structure of ATP-bound Get3-Get4-Get5 complex reveals regulation of Get3 by Get4. *Nat Struct Mol Biol* 21(5):437-442.
18. Mock JY*, et al.* (2015) Bag6 complex contains a minimal tail-anchor-targeting module and a mock BAG domain. *Proc Natl Acad Sci U S A* 112(1):106-111.