Supporting Information

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SI Materials and Methods

Yeast Two-Hybrid Assay. The PJ69-4α strain was obtained from the Yeast Resource Center at the University of Washington. Bag6 fragments A (residues 1–225), B (residues 226–399), C (residues 400–659), D (residues 660–950), E (residues 951–1,126), Bag6E_N (residues 951–1,011), Bag6E_{NLS} (residues 1,012–1,054), and Bag6E_{BAG} (residues 1,055–1,126) were cloned into the pGAD-C1 vector. TRC35, TRC35-N (residues 1–157), TRC35-C (residues 158–327), and Ubl4A were cloned into the pGBDU-C1 vector. Alanine mutants were made using site-directed mutagenesis (Agilent Technologies). pGAD-C1 and pGBDU-C1 containing genes of interest were cotransformed into PJ69-4α using previously described methods (1) and then were plated on SC-Ura-Leu and incubated at 30 °C. The double transformants then were inoculated into 5 mL SC-Ura-Leu and grown overnight in a shaking incubator (Multitron Standard Infors HT) at 200 rpm at 30 °C. From the cultures 2×10^7 cells were reinoculated into 5 mL total SC-Ura-Leu and grown in a shaker incubator for 6 h. Cells were harvested by spinning at $3,000 \times g$ at room temperature and were washed twice with 5 mL sterile water. Then 1×10^7 cells were resuspended in 40 μL of sterile water, and 4 μL of this resuspended sample was spot-plated onto SC-Ura-Leu-Ade and grown for 72 h at 30 °C.

Cloning, Expression, and Purification. For crystallization, 54 residues from Bag6 (Q1054–D1107) and the 53 C-terminal residues from Ubl4A (P93–P145) were coexpressed with pET33b plasmid with the N-terminal 6xHis tag on Ubl4A-C. To facilitate tobacco etch virus (TEV) cleavage of the histidine tag, three flexible residues, glycine, alanine, and serine, were inserted between the TEV cut site and P93 of Ubl4A using site-directed mutagenesis. The proteins were expressed in *E. coli* NiCo21(DE3) (New England Biolabs) for 3 h at 37 °C after induction with 300 μM isopropyl-β-D-thiogalactopyranoside (Affymetrix). Cells were lysed using an S-4000 sonicator (Misonix) in 50 mM NaH_2PO_4, 200 mM NaCl, 20 mM imidazole supplemented with benzamidine, PMSF, and 0.5% Triton X-100. The complex was purified by Ni-NTA affinity chromatography (Qiagen) and then was incubated overnight at room temperature with TEV protease in 20 mM NaH_2PO_4, 100 mM NaCl, 20 mM imidazole, and 10 mM β-mercaptoethanol followed by size-exclusion chromatography on a Superdex 75 gel-filtration column in 10 mM Tris, 50 mM NaCl (pH 8.0) and was concentrated to ∼10 mg/mL.

All truncations of Bag6 were purified using the protocol described above. The 53 C-terminal residues from L1055 to D1107 of Bag6 were cloned for Bag6-BAG. The Bag6/Ubl4A construct contained the 73 C-terminal residues and full-length Ubl4A. The 175 C-terminal residues from V951 to P1126 were cloned in the Bag6 E fragment vector. Bag6-C81 contained the 81 C-terminal residues from K1046 to P1126.

cDNA of human Hsc70, Hdj1, and Hsc70-NBD (residues P5 to S381) were subcloned into pET33b vector, expressed, and purified as previously described (2), with some modifications. Full-length Hsc70 and Hsc70-NBD were purified over a UnoQ column (Bio-Rad) [50 mM Hepes, 50–500 mM KCl gradient (pH 8.0), 5 mM β-mercaptoethanol]. Hdj1 was purified over a UnoS column [50 mM Hepes, 20–500 mM KCl gradient, 500 mM KCl (pH 7.0), 5 mM β-mercaptoethanol]. The plasmid vectors containing the cDNA of the chaperone proteins were obtained from the Morimoto group at Northwestern University, Evanston, IL (3).

cDNA of human TRC40 was subcloned into the pGEX-6P-1 vector and was expressed in NiCo21(DE3) cells. Cells were lysed using an M-110L Microfluidizer Processor (Microfluidics) in 50 mM NaH_2PO_4 and 400 mM NaCl supplemented with benzamidine, PMSF, and 5 mM β-mercaptoethanol. The protein was purified in single-step glutathione SuperFlow resin affinity chromatography (Clontech).

cDNA of human SGTA was subcloned into the pET33b vector and was coexpressed with MBP-Sbh1 in the pACYCDuet vector in NiCo21(DE3) cells. Cells were lysed using an M-110L Microfluidizer Processor (Microfluidics) in 50 mM NaH_2PO_4, 150 mM NaCl, 20 mM imidazole supplemented with benzamidine, PMSF, and 5 mM β-mercaptoethanol. The complex was purified in two steps using Ni-NTA resin (Qiagen) and amylose resin (New England Biolabs).

Human TRC35(23–305) was subcloned from TRC35 cDNA into the pACYCDuet vector in the first multiple cloning site (MCS) with an N-terminal 6xHis tag and a TEV protease cut site. In the second MCS, cDNA of untagged human Ubl4A was subcloned. The subcloned TRC35 fragment in pACYCDuet was coexpressed with untagged Bag6(1,001–1,126) in pET33b in NiCo21(DE3). The complex was purified by Ni-NTA affinity chromatography (Qiagen). Contaminants were removed further using chitin affinity chromatography (New England Biolabs). TRC40(K86D), TRC35(D84K), SGTA(C38A), and Ubl4A(L43A) mutants were generated using site-directed mutagenesis (Agilent Technologies).

Circular Dichroism. Circular dichroism spectra were obtained using an Aviv 62A DS circular dichroism spectrometer. The ellipticity of 10 μM of Bag6-BAG, Ubl4A-C, or Bag6/Ubl4A suspended in 10 mM Tris, 50 mM NaCl (pH 8.0) was measured.

Crystallization. Crystallization screening was performed using the sitting-drop vapor-diffusion method with commercially available screens (Hampton) and was set up by a Mosquito robot (TTP Labtech) and incubated at room temperature. The heterodimer crystallized after 4 d as rectangular prisms in 20% (wt/vol) PEG-3350, 0.05 M Hepes (pH 7.0), 1% tryptone (Hampton). The crystals were soaked in 20% (vol/vol) glycerol for 15 s and cryopreserved in liquid nitrogen. Iodide derivatives were generated by soaking crystals in 40 μL mother liquor [20% (wt/vol) PEG-3350, 0.05 M Hepes (pH 7.0), 1% tryptone (Hampton)], 10 μL ethylene glycol, and 3 μL 6 M sodium iodide for 2–10 s before cryopreservation.

Data Collection, Structure Solution, and Refinement. X-ray diffraction data were collected on Beam Line 8.2.1 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory. A complete dataset was collected from a single crystal to 2.0-Å resolution. Data were integrated, scaled, and merged using MOSFLM (4) and XDS (5), and the data were merged and converted with SCALA (6, 7). Phases were determined by single-wavelength anomalous dispersion data merged from three iodide derivative crystals, which diffracted to 3.5 Å. The model was obtained by PHASER (8) and RESOLVE (9) as implemented automatically by PHENIX (10) and further refined against the native dataset using COOT (11) for manual building and PHENIX (10). The final model contained four molecules in the asymmetric unit with three of the four heterodimers having an rmsd of 0.382 accounting for residues 97–128 of Ubl4A and 1,058–1,102 of Bag6. The final heterodimer, molecules F and G (rmsd = 0.675 when included), appeared to have a slightly altered conformation of the second helix in the Ubl4A dimerization domain that could not be fully resolved; however, there was no significant change to the overall architecture.

β-Galactosidase Refolding Assay. The Hsc70-mediated β-galactosidase refolding assay was carried out as previously reported (3) with modifications. A stock solution of β-galactosidase at 10 mg/mL was prepared by dissolving the enzyme (Sigma-Aldrich) in 50 mM Tris·HCl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol (pH 7.3). For experiments, the stock enzyme was diluted 1:10 in 1 M glycylglycine (pH 7.4); 5 μL of this stock was diluted into 95 μL of unfolding buffer [25 mM Hepes, 5 mM $MgCl_2$, 50 mM KCl, 5 mM mercaptoethanol, 6 M guanidine-HCl (pH 7.4)], and 5 μL was diluted into 95 μL 1 M glycylglycine (pH 7.4) for the control. The final β-galactosidase concentration was 3.4 nM. The two samples were incubated at 30 °C for 30 min.

Folding reactions were performed in refolding buffer [1.6 μM Hsc70 and 3.2 μM Hdj1 suspended in 25 mM Hepes, 5 mM $MgCl_2$, 50 mM KCl, 2 mM ATP, 10 mM DTT (pH 7.4)]. Varying concentrations of Bag6 constructs were tested for their effect on Hsc70 folding activity. After 30-min incubation at 30 °C, 1 μL of denatured enzyme was added to 124 μL of each refolding reaction tube and incubated at 37 °C. At regular time intervals, 10 μL of each folding reaction was added to 10 μL of 0.8 mg/mL ortho-nitrophenyl-β-galactoside (ONPG) and incubated at 37 °C for 15 min. The reaction was stopped by the addition of 80 μL 0.5 M sodium carbonate. β-Galactosidase activity was measured as the rate of conversion of ONPG by absorbance at 413 nm.

Hsp/Hsc70 Capture Assays. Five hundred picomoles of Hsc70-NBD with an N-terminal 6xHis tag were incubated with 2 nmol of untagged Bag6-BAG, Bag6-C81, $Bag6E_{BAG}$/Ubl4A, and Bag1-BAG in a total of 100 μL of binding buffer (20 mM Hepes, 100 mM KCl, 20 mM imidazole, and 5 mM β-mercaptoethanol) for 1 h at room temperature. The samples then were added to 15 μL of Ni-NTA beads (Qiagen). The beads were washed twice with 100 μL of binding buffer. Bound proteins were eluted with 15 μL of 20 mM Hepes, 100 mM KCl, 300 mM imidazole, and 5 mM β-mercaptoethanol and then were run on a 4–20% gradient SDS/PAGE gel (Bio-Rad). Four percent of the total sample was run as loading control.

For capture assays from 293T whole-cell lysate, cells from a 10-cm dish (90% confluent) were lysed in 1.5 mL Nonidet P-40 lysis buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40, 2 mM β-mercaptoethanol, protease inhibitor mixture]. Cell extracts were subject to centrifugation at 20,000 × *g* for 5 min to remove insoluble materials. The soluble fractions were pretreated with 180 mL HisPur Coblat resin (Thermo) and then were incubated with 30 mL HisPur Coblat resin immobilized with histidine-tagged Bag6-fragments at 4 °C for 1 h, as indicated in Fig. 5*B*. The resins were washed twice quickly with 400 mL of a wash buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM $MgCl_2$], 0.1% Nonidet P-40, and 2 mM β-mercaptoethanol. The proteins bound to the resin were eluted with 60 mL SDS/PAGE loading buffer and denatured by heating at 65 °C for 10 min. The samples were analyzed by SDS/PAGE. Proteins were detected by either Ponceau staining and immunoblotting.

To examine the in vivo interaction of Bag6 and Hsp70, cells grown in a six-well plate were transfected with plasmids expressing FLAG-tagged wild-type Bag6 or mutant Bag6 lacking the 81 C-terminal amino acids using TransIT 293 (Mirus). Cells were lysed in the Nonidet P-40 lysis buffer 24 h post transfection. Bag6 was pulled down from the lysate by FLAG M2 beads (Sigma-Aldrich). The precipitated material was analyzed by immunoblotting.

In Vitro TA Handoff Assay. hSGTA/MBP-Sbh1 (6.25 pmol) or hSGTA(C38A)/MBP-Sbh1 (6.25 pmol) was incubated with 62.5 GST-TRC40 or GST-TRC(K86D) with or without 0.008 μg/μL of $Bag6_{min}$, $Bag6_{min}$(D84K), or $Bag6_{min}$(L43A) complex in 100 μL of incubation buffer [50 mM Hepes (pH 7.5), 4 mM Mg(OAc)2, 150 mM KOAc, 10% glycerol, and 1 mM DTT] on ice for 10 min. After 10 min, 10 μL of MagneGST resin (Promega) was added to each reaction and incubated at room temperature for 15 min to pull down GST-TRC40 and bound factors. The resin was washed three times with 500 μL of incubation buffer and eluted with 20 μL of 20 mM Tris, 300 mM NaCl, and 33 mM L-glutathione (pH 7.4). The precipitated material was analyzed by immunoblotting. For the wild-type capture experiment, ~12 ± 5.2% of TRC40 and 4.1 ± 1.6% of Sbh1 were eluted from the beads. One would expect ~12% of the TA to be captured assuming 100% transfer; therefore, our yield is ~25%. Differences could be attributed to a variety of factors such as differences in the affinities of the various TRC40 complexes.

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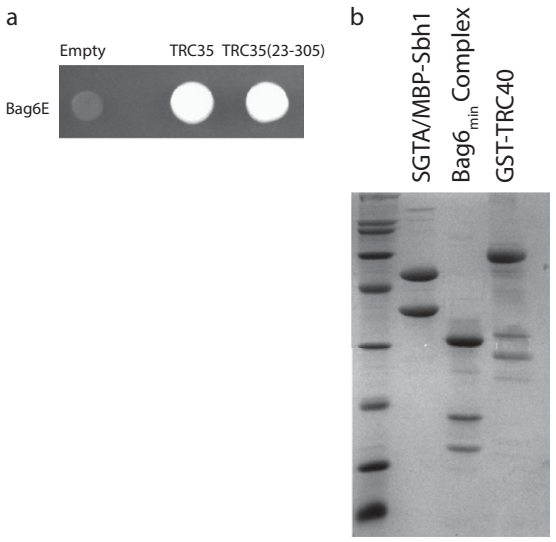

Fig. S1. Purification of functional recombinant proteins used in the in vitro TA transfer assay. (*A*) Two-hybrid assay using full-length TRC35 and TRC35(23–305) with the Bag6E fragment. Both TRC35 constructs display strong two-hybrid interactions. (*B*) Representative Coomassie-stained 12% SDS/PAGE gel of purified hSGTA/MBP-Sbh1, $Bag6_{min}$ complex, and GST-TRC40.

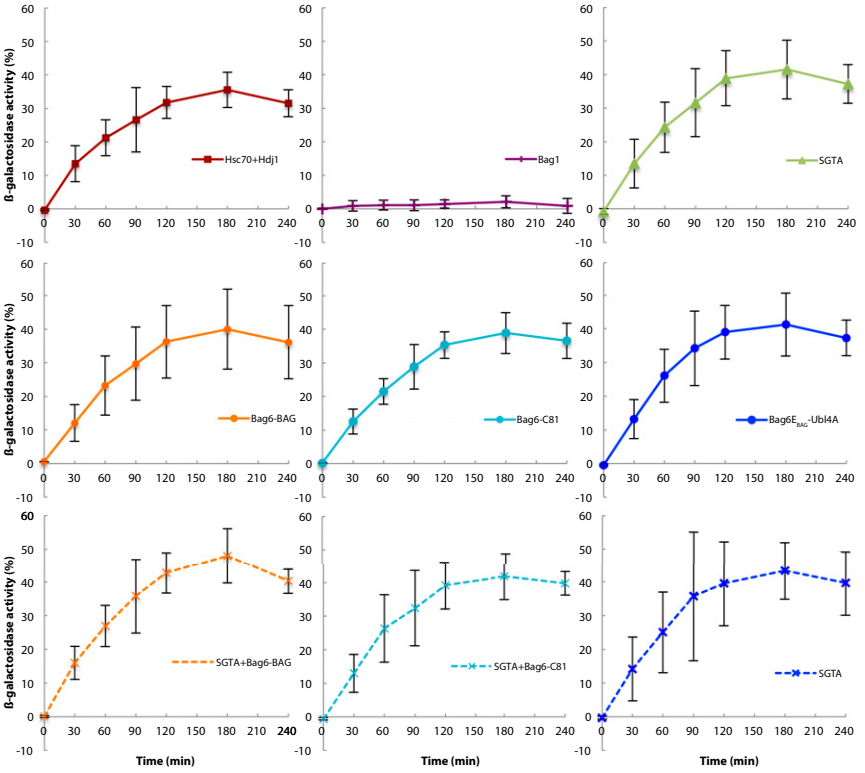

Fig. S2. Individual results for various in vitro refolding assays. β-Galactosidase refolding assays in the presence of Hsc70, Hdj1, and/or other factors as labeled. Colors are based on Fig. 5 except for assays containing Bag6-C81, which are in cyan. Error bars are from three independent experiments.

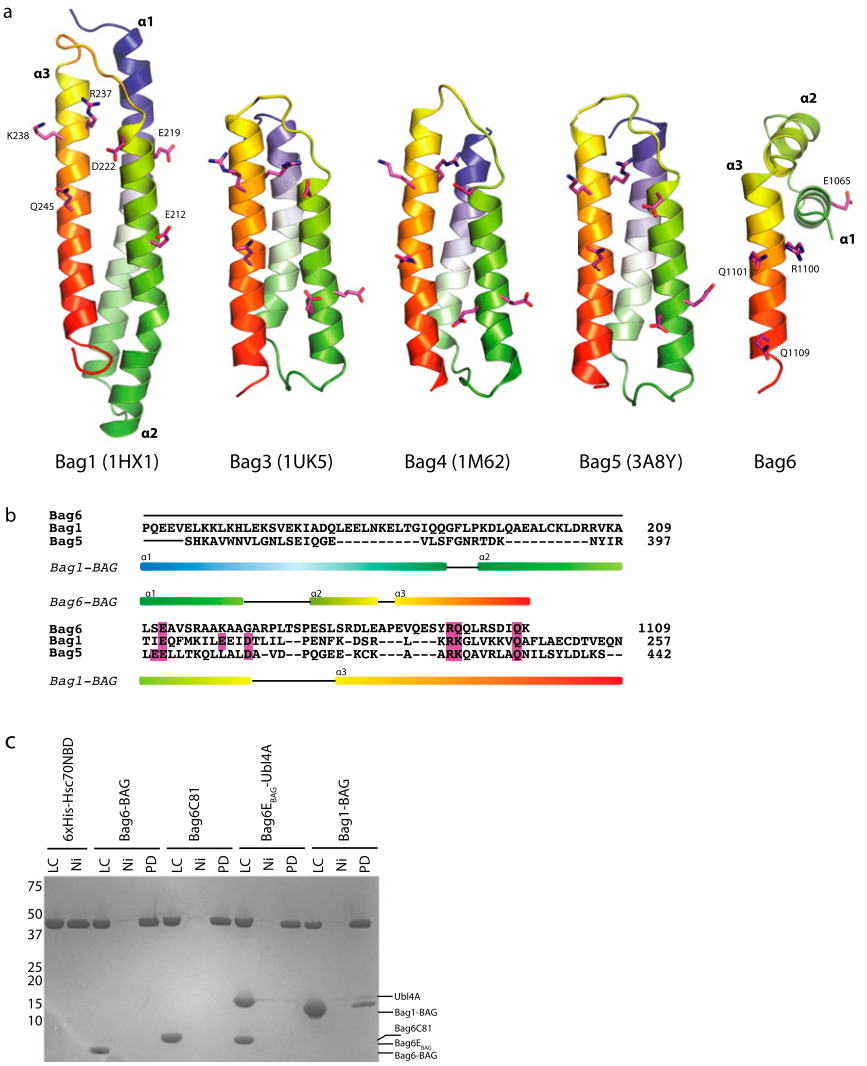

Fig. S3. A comparison of Bag6-BAG and canonical BAG domains. (*A*) Published structures of BAG domains are shown as ribbons similar to Bag6-BAG. Residues involved in Hsp/Hsc70 binding are highlighted as magenta sticks. (*B*) Sequence alignment of human Bag1-BAG, Bag5-BAG5, and Bag6-BAG with the known secondary structure indicated. (*C*) In vitro capture by 6xHis-Hsc70-NBD of Bag6-BAG, Bag6-C81, Bag6E$_{BAG}$/Ubl4A, or Bag1-BAG. Protein was pulled down (PD) with Ni-NTA after incubation with 6xHis-Hsc70-NBD. Four percent of total loaded protein is shown as a loading control (LC), and each protein was incubated alone with Ni-NTA (Ni) to assess background binding.

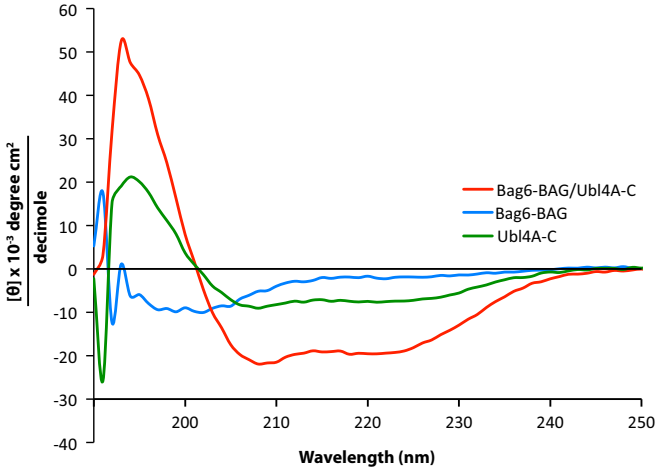

Fig. S4. Circular dichroism spectra of Bag6-BAG (blue), Ubl4A-C (green), and the complex of the two (red).

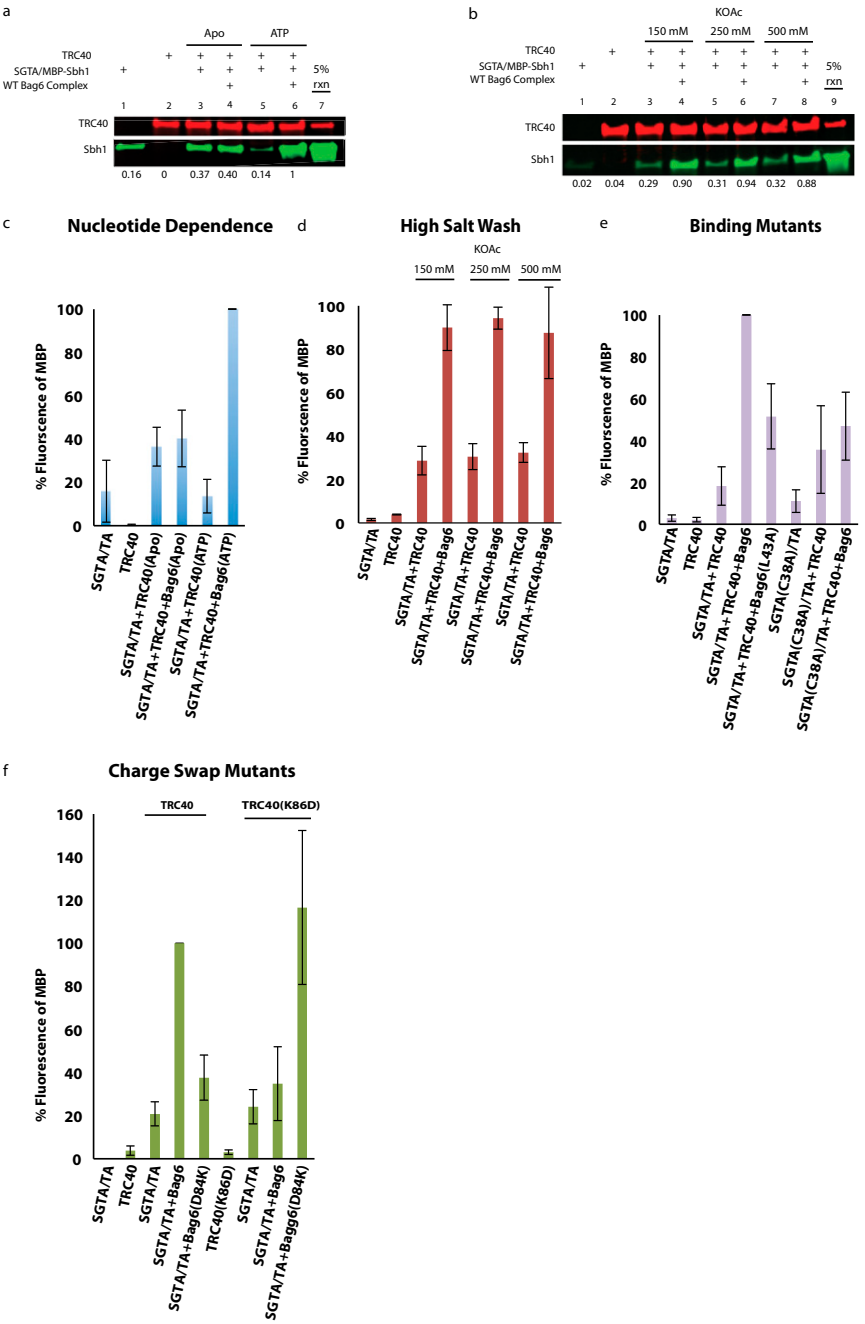

Fig. S5. TA handoff from SGTA to TRC40. (*A*) Nucleotide-dependent TA handoff facilitated by $Bag6_{min}$ complex. GST-TRC40 was captured on anti-GST resin after incubation with SGTA-MBP/Sbh1 and $Bag6_{min}$ complex with or without ATP. Eluted samples were immunoblotted with anti-GST (red) and anti-MBP antibody (green) and then were quantified by Odyssey Infrared Imaging System analysis software. The Sbh1 values were normalized based on total GST-TRC40 captured. The MBP-Sbh1 signal from the wild-type experiment was designated 1, and the rest are represented as a fraction of the wild-type value. Values are average of four independent experiments. (*B*) TA handoff from SGTA to TRC40 facilitated by the $Bag6_{min}$ complex in the presence of ATP washed with buffers with varying salt concentrations and analyzed as in *A*. Values are average of three independent experiments. (*C*) Average values of nucleotide-dependent TA handoff from SGTA to TRC40 facilitated by the $Bag6_{min}$ complex. Error bars are from four independent experiments. (*D*) Average values of TA handoff from SGTA to TRC40 facilitated by the $Bag6_{min}$ complex in the presence of ATP washed with buffers with varying salt concentrations. Error bars are from three independent experiments. Fluorescence values are represented as a percentage of the wild-type handoff as measured by MBP fluorescence. (*E*) Average values of TA handoff by binding mutants hSGTA(C38A)/MBP-Sbh1 and $Bag6_{min}$Ubl4A(L43A) complex compared with wild type. Error bars are from six independent experiments. (*F*) Average values of TA handoff of regulatory mutants GST-TRC40(K86D) and $Bag6_{min}$TRC35(D84K) complex compared with wild type. Error bars are from four independent experiments.

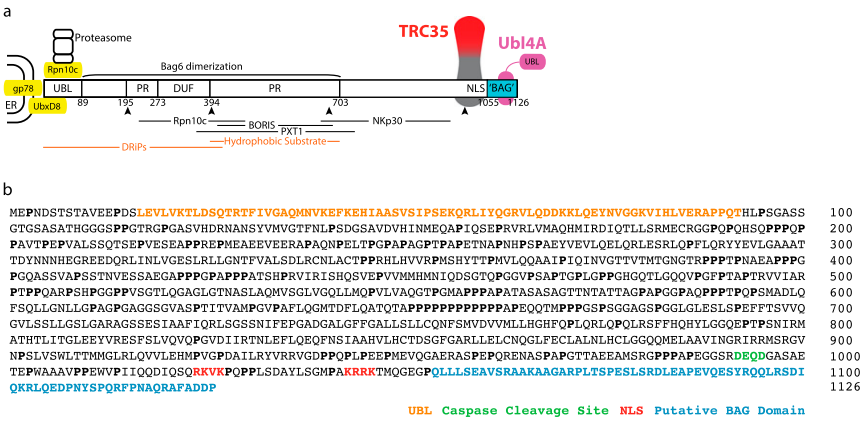

Fig. S6. The Bag6 complex is a scaffold for a broad range of activities. (*A*) A model of the Bag6 complex and its defined interactions. For Bag6, the locations of the UBL (PDB ID codes 4EEW, 4DWF, and 1WX9), proline-rich domains (PR), domain of unknown function (DUF) (1), NLS (2), and the BAG domain (cyan) are shown. Arrows indicate the five fragments of Bag6 (A, B, C, D, E) used in this study. Regions required for interaction with RP non-ATPase 10c (Rpn10c) (3), BORIS (4), PXT1 (5), and NKp30 (6) are indicated by black lines. The regions required for binding of hydrophobic substrates and polyubiquitinated defective ribosomal products (DRiPs) (7) are displayed in orange. The region required for Bag6 dimerization (1) is indicated on top. Proteins with defined interactions are shown as colored boxes. Bag6-UBL–binding proteins are highlighted in yellow. The membrane-embedded ubiquitin regulatory X domain-containing protein 8 (UbxD8) and transmembrane protein gp78 (1) are thought to anchor the Bag6 complex to the ER. Rpn10c is a component of the proteasome. For the Bag6 complex, TRC35 (red and gray) and Ubl4A (magenta) are shown with their domains indicated. (*B*) Amino acid sequence of the Bag6 isoform used in this study.

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Table S1. Crystallographic analysis

	Native	Iodide
Data collection		
Beamline	ALS 8.2.1	ALS 8.2.1
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, *b*, *c*, Å	47.2, 56.4, 75.5	47.4, 57.2, 75.9
α, β, γ, °	90.0, 96.2, 90.0	90.0, 95.9, 90.0
Wavelength	1.0000	1.7000
Resolution, Å	19.65–2.00 (2.07–2.00)	28.6–2.70 (2.80–2.70)
R_{merge}, %*	4.68 (74.85)	14.2 (162.6)
$< I >/< \sigma I >$*	15.38 (1.89)	24.8 (3.0)
Completeness, %*	98.48 (97.81)	99.9 (100.0)
No. of observations	78,009 (7,708)	258,426 (26,352)
No. of unique reflections*	26,486 (2,592)	11,261 (1,127)
Multiplicity	2.9 (3.0)	22.9 (23.3)
Refinement		
No. of reflections	25,149	
No. of reflections test set	1,334	
R_{work} (%)/R_{free} (%)	22.45 (33.11)/26.88 (40.64)	
No. atoms (nonhydrogen)	3,296	
Protein	3,167	
Water	129	
Average *B*-factor	54.60	
RMSD		
Bond lengths, Å	0.007	
Bond angles, °	1.07	
Ramachandran plot		
Favored, %	97.9	
Additionally allowed, %	1.8	
Outliers, %	0.3	

*Highest-resolution shell is shown in parentheses.