SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

i/TIF34-b/PRT1 Affinity Analysis Using Gel Mobility Shift Assays.

The [35S]-labeled C-terminal b/PRT1 peptides were prepared using the TnT coupled rabbit reticulocyte in vitro translation and transcription system (Promega). Proteins were synthesized by incubating 1 µg of plasmid in 40 µl of TnT Quick Master mixture with 2 µl of L-[35S] methionine (Amersham Biosciences) for 2 hours at 25°C. The in vitro translated products were then purified using HIS-Select™ spin columns (Sigma) according to the manufacturer's instructions. Unlabeled C-terminal b/PRT1 peptides and i/TIF34 were expressed in E. coli and purified as above. Dissociation constant of b/PRT1(630-724) was determined by titrating purified [35S]-b/PRT1 (0.1 µM final concentration) with increasing amount of purified i/TIF34 (0, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 25.0 µM final concentration) in a final volume of 20 µl reaction mix containing 20 mM Hepes pH 7.5, 50 mM NaCl and 5% glycerol. For competition assay, 0.2 µM [35S]-b/PRT1 (630-724) was first mixed with 1 µM i/TIF34, then unlabeled b/PRT1 peptides were added to increasing concentrations at the following ratios of unlabelled to labelled protein: 0.1, 0.5, 1, 10, 25, 50, 100 for b(630-724); 1, 2, 10, 25, 50, 100 for W674F; 1, 10, 25, 50, 100, 200 for W674A; 1, 10, 25, 50, 200 for Y677A/R68A; and 1, 5, 50, 100, 200 for b(654-700). Complexes were formed at 30°C and analyzed on 4-16% native polyacrylamide BisTris gels (Invitrogen). Gel electrophoresis was performed for 2 h at 100 V at 4 °C using NativePAGE running buffer (Invitrogen). Gels were then dried on Whatman paper and exposed overnight on a PhosphorImager screen. Bound and free radioactive b/PRT1 bands were quantified using ImageQuantTL image analysis tool. Data analysis was performed using DynaFit software (58,59). Direct and competition titration data were fit using equilibrium binding or displacement models for single site binding.

Calorimetry (ITC) Analysis of i/TIF34-b/PRT1 Interactions.

All calorimetric titrations were performed on a VP-ITC microcalorimeter (Microcal). Protein samples were dialyzed against the same storage/NMR buffer (see Materials and Methods in the main text). The sample cell was filled with 200 µl of 26 µM solution of i/TIF34 and the injection syringe with 288 µM or 200 µM of b/PRT1(654-700) or b/PRT1(630-724), respectively. Each titration typically consisted of a preliminary 0.5 µl injection followed by 20 subsequent 2-µl injections every 120 seconds. All of the experiments were performed at 25 °C. Data for the preliminary injection, which are affected by diffusion of the solution from and into the injection syringe during the initial equilibration period, were discarded. Binding isotherms were generated by plotting heats of reaction normalized by the moles of injectant versus the ratio of total injectant to total protein per injection. The data were fitted to single site binding model using Origin 7.0 (Microcal).
Yeast Genetic and Biochemical Methods.
GST pull-down assays with GST fusions and in vitro-synthesized \(^{[35]S}\)-labeled polypeptides were conducted as follows. Individual GST-fusion proteins were expressed in *E. coli*, immobilized on glutathione-Sepharose beads and incubated with 10 \(\mu\)l of \(^{[35]S}\)-labeled potential binding partners at 4°C for 2 h. The beads were washed 3 times with 1 ml of phosphate-buffered saline and bound proteins separated by SDS-PAGE. Gels were first stained with Gelcode Blue Stain Reagent (Pierce) and then subjected to autoradiography. Ni\(^{2+}\)-chelation chromatography of eIF3 complexes containing His\(_8\)-tagged b/PRT1 or g/TIF35 from yeast whole-cell extracts (WCEs) and Western blot analysis were conducted as described in detail previously (60). In short, WCEs were incubated with 15 \(\mu\)L of 50% Ni\(^{2+}\)-NTA-silica resin (GE Healthcare) suspended in 200 \(\mu\)L of buffer A for 2 h at 4°C, followed by washing and elution. 2% HCHO cross-linking followed by WCE preparation and fractionation of extracts for analysis of pre-initiation complexes were carried out as described previously (38). \(\beta\)-galactosidase assays were conducted as described previously (61).

Preparation of Antibodies Against RPS0A.
The GST-RPS0A fusion protein encoded by pGEX-RPS0A was expressed in *E. coli* and purified from the WCE by incubation with Glutathione-Sepharose 4B beads (Pharmacia). The isolated protein was resolved by SDS-PAGE (4-20% gels), excised from the gel, and washed with 1x PBS. Rabbits were injected with the purified protein and serum containing polyclonal antibodies against RPS0A was obtained commercially by Apronex (Prague, the Czech Republic).

Construction of Yeast Strains and Plasmids.
List of all strains named below can be found in Tables S3.
To create AY134 strain, YAH06 was transformed with pRS-b/PRT1-HisXS and the original covering plasmid carrying *PRT1 URA3* was evicted on 5-FOA.
YAH11 was generated by a genetic cross of H420 and AY134 as a haploid ascospore resistant to 3-AT, unable to grow on 5-FOA, unable to evict a resident *LEU2* covering plasmid, and autotrophic for tryptophan.
To create YAH12 strain, YAH11 was transformed with pCR52-1 (62) and grown on SD media containing Leucine to get rid of the original pRS-b/PRT1-HisXS plasmid.
List of all plasmids and PCR primers named below can be found in Tables S4 and S5, respectively.
pRS-b/PRT1-W674A-His was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using pRS-b/PRT1-His as template: (1) AH-PRT1-BamHI and AH-PRT1-W674A-R, respectively, (2) AH-PRT1-W674A and AH-PRT1-NotI-R, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers AH-PRT1-BamHI and AH-PRT1-NotI-R. The resulting PCR product was digested with BamHI and NotI and ligated with BamHI-NotI-cleaved pRS-b/PRT1-His.
pRS-b/PRT1-W674F-His – was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using pRS-b/PRT1-His as template: (1) AH-PRT1-BamHI and AH-PRT1-W674F-R, respectively, (2) AH-PRT1-W674F and AH-PRT1-NotI-R, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers AH-PRT1-BamHI and AH-PRT1-NotI-R. The resulting PCR product was digested with BamHI and NotI and ligated with BamHI-NotI-cleaved pRS-b/PRT1-His.

pRS-b/PRT1-Y677A-His was made by inserting the BamHI-MscI digested PCR product obtained with primers AH-PRT1-BamHI and AH-PRT1-Y677A-R using pRS-b/PRT1-HisXS as a template into BamHI-MscI digested pRS-b/PRT1-HisXS.

pRS-b/PRT1-R678A-His was made by inserting the BamHI-MscI digested PCR product obtained with primers AH-PRT1-BamHI and AH-PRT1-R678A-R using pRS-b/PRT1-HisXS as a template into BamHI-MscI digested pRS-b/PRT1-HisXS.

pRS-b/PRT1-Y677A-R678A-His was made by inserting the BamHI-MscI digested PCR product obtained with primers AH-PRT1-BamHI and AH-PRT1-YR-AA-R using pRS-b/PRT1-HisXS as a template into BamHI-MscI digested pRS-b/PRT1-HisXS.

pRS-b/PRT1-Y677A-R678D-His was made by inserting the BamHI-MscI digested PCR product obtained with primers AH-PRT1-BamHI and AH-PRT1-YR-AD-R using pRS-b/PRT1-HisXS as a template into BamHI-MscI digested pRS-b/PRT1-HisXS.

YCp-i/TIF34-D207K-HA was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using YCp-i/TIF34-HA as template: (1) y3iKpnI and D207Kr, respectively, (2) D207K and BmgBlr, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers y3iKpnI and BmgBlr. The resulting PCR product was digested with KpnI and BmgBlr and ligated with KpnI-BmgBlr-cleaved YCp-i/TIF34-HA.

YCp-i/TIF34-D224K-HA was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using YCp-i/TIF34-HA as template: (1) y3iKpnI and D224Kr, respectively, (2) D224K and BmgBlr, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers y3iKpnI and BmgBlr. The resulting PCR product was digested with KpnI and BmgBlr and ligated with KpnI-BmgBlr-cleaved YCp-i/TIF34-HA.

YCp-i/TIF34-D207K-D224K-HA was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using YCp-i/TIF34-D207K-HA as template: (1) y3iKpnI and D224Kr, respectively, (2) D224K and BmgBlr, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers y3iKpnI and BmgBlr. The resulting PCR product was digested with KpnI and BmgBlr and ligated with KpnI-BmgBlr-cleaved YCp-i/TIF34-D207K-HA.
YCp-i/TIF34-L222D-HA was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using YCp-i/TIF34-HA as template: (1) y3iKpnI and LC3iLr, respectively, (2) LC3iL222D and BmgBIr, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers y3iKpnI and BmgBIr. The resulting PCR product was digested with KpnI and BmgBI and ligated with KpnI-BmgBI-cleaved YCp-i/TIF34-HA.

YCp-i/TIF34-L222K-HA was generated by fusion PCR. The following pairs of primers were used for separate PCR amplifications using YCp-i/TIF34-HA as template: (1) y3iKpnI and LC3iLr, respectively, (2) LC3iL222K and BmgBIr, respectively. The PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers y3iKpnI and BmgBIr. The resulting PCR product was digested with KpnI and BmgBI and ligated with KpnI-BmgBI-cleaved YCp-i/TIF34-HA.

YCp-i/TIF34-HA-W was constructed by inserting the 2223bp SacI-DrdI fragment from YCp-i/TIF34-HA into YCplac22 digested with SacI-DrdI.

YCp-i/TIF34-D207K-D224K-HA-W was constructed by inserting the 2223bp SacI-DrdI fragment from YCp-i/TIF34-D207K-D224K-HA into YCplac22 digested with SacI-DrdI.

YCp-i/TIF34-D207K-HA-W was constructed by inserting the 2223bp SacI-DrdI fragment from YCp-i/TIF34-D207K-HA into YCplac22 digested with SacI-DrdI.

YCp-i/TIF34-D224K-HA-W was constructed by inserting the 2223bp SacI-DrdI fragment from YCp-i/TIF34-D224K-HA into YCplac22 digested with SacI-DrdI.

YEp-g/TIF35-His was constructed by inserting the 2112bp EcoRI-PstI fragment from YCp-g/TIF35-His-screen into YEplac195 digested with EcoRI-PstI.

YEp-SUI1-G107R was constructed by inserting the 287bp BamHI-SacII fragment from pCFB134 into YEp-SUI1 digested with BamHI-SacII.

pT7-b/PRT1-W674A was constructed by inserting the 1222bp NsiI-MscI fragment from pRS-b/PRT1-W674A-His into pT7-b/PRT1 digested with NsiI-MscI.

pT7-b/PRT1-Y677A was constructed by inserting the 1222bp NsiI-MscI fragment from pRS-b/PRT1-Y677A-His into pT7-b/PRT1 digested with NsiI-MscI.

pT7-b/PRT1-R678A was constructed by inserting the 1222bp NsiI-MscI fragment from pRS-b/PRT1-R678A-His into pT7-b/PRT1 digested with NsiI-MscI.

pT7-i/TIF34-D207K-D224K was constructed by inserting the 587bp KpnI-EcoRI fragment from YCp-i/TIF34-D207K-D224K-HA into pT7-i/TIF34 digested with KpnI-EcoRI.

pGEX-RPS0A was constructed in two steps. First, the intron was removed by a fusion PCR using pGBK-RPS0A (37) as a template and the following pair of primers for two separate PCR reactions producing exon 1 (O1-BamHI x BSRPS0Aexon1-r) and exon2 (BSRPS0Aexon2 x O2-KpnI-XhoI-r), respectively. Both PCR products thus obtained were used in a 1:1 ratio as templates for a third PCR amplification using primers O1-BamHI and O2-KpnI-XhoI-r. The resulting PCR product was digested with KpnI and BmgBI and ligated with KpnI-BmgBI-cleaved YCp-i/TIF34-HA.
product was digested with BamHI and XhoI and ligated with BamHI-XhoI-cleaved pGEX-4T-1 (63).

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FIGURE LEGENDS

Figure S1. Identification of the minimal i/TIF34-binding site of b/PRT1. (A) Overlay of $^1$H, $^{15}$N-TROSY (transverse relaxation optimized spectroscopy) spectra of $^{13}$C, $^{15}$N-labelled b/PRT1(630-724) in the free form (purple) or bound to unlabelled i/TIF34 (pink). Assigned amide crosspeaks from bound b/PRT1(630-724) which display sharp resonance lines and random coil chemical shifts are labeled. The NeH side chain crosspeaks of W644 and W674, which resonate downfield of the backbone amide NH crosspeaks are shown in an inset. Only the W674-NeH resonances display large chemical changes upon i/TIF34 binding, while W644-NeH remains unchanged suggesting that the middle part if b/PRT1(630-724) comprises the i/TIF34 binding site. (B) Overlay of $^1$H, $^{15}$N-TROSY spectra of $^{15}$N-labelled b/PRT1(630-724; purple) or $^{15}$N-labelled b/PRT1(654-700; pink) both bound to unlabelled i/TIF34. The shorter b/PRT1 peptide lacks most of the sharp, random coil amide NH resonances from the longer b/PRT1 peptide, while very similar, broad amide NH resonances close to the noise level arising from i/TIF34-interacting parts of b/PRT1 are observed in both peptides. The Trp-NeH side chain crosspeaks are shown in an inset. The shorter b/PRT1 peptide (pink) lacks the sharp, random coil W644-NeH resonances of the longer peptide (purple), but displays the same broad, downfield-shifted crosspeak for W674-NeH as found for the longer peptide suggesting a very similar binding mode of both peptides. (C) Isothermal calorimetric titration of i/TIF34 with b/PRT1(630-724) and b/PRT1(654-700) reveal comparable binding affinities. The panels show experimental data and the fitted binding isotherms. The data was analyzed using Origin 7.0 (MicroCal) software. Data points were obtained by integration of heat signals plotted against the molar ratio of i/TIF34 to b/PRT1 variants in the reaction cell. The solid line represents a calculated curve using the best-fit parameters obtained by a nonlinear least squares fit. The b/PRT1 construct used for each experiment is indicated in the panel.

Figure S2. Sequence alignment of i/TIF34 and b/PRT1 from different species and stereo view of the i/TIF34 - b/PRT1 binding interface. (A) Sequence alignment of eIF3i/TIF34 and eIF3b/PRT1 from S. cerevisiae, human, Drosophila, frog, Neurospora, Mouse and Arabidopsis. Phylogenetic analysis reveals a high level of
eIF3i/TIF34 and eIF3b/PRT1 conservation between species. In eIF3b/PRT1, only the C-terminus is shown; blue triangles indicate boundaries of our eIF3b/PRT1 construct, residues important for the interaction and discussed in the text are numbered according to S. cerevisiae. Secondary structure elements are shown above the aligned sequences. (B) Interface of i/TIF34 and b/PRT1(654-700) interactions. i/TIF34 is in green, b/PRT1(654-700) in blue. Cross-eye stereo image was generated using PyMOL (http://www.pymol.org).

**Figure S3.** Structure of the i/TIF34-b/PRT1(654-700) complex. (A) Left, experimentally phased, solvent-flattened electron density map with the refined model superimposed. Right, part of the experimental electron density map in the region of b/PRT1(654-700). Protein backbones are shown as ribbons. (B) Overall view of the i/TIF34-b/PRT1(654-700) complex (two complexes in asymmetric unit) with the corresponding 2mFo-DFc electron density map shown contoured at 1 sigma. i/TIF34 is in green, b/PRT1(654-700) in blue. Ray-traced images were generated using PyMOL (http://www.pymol.org).

**Figure S4.** Two i/TIF34-b/PRT1 complexes are present in the asymmetric unit. i/TIF34 chains are shown in green, b/PRT1 in blue. Blades in the β-propeller structure of i/TIF34 are numbered from 1 to 7 (N to C-terminus). The two complexes in the asymmetric unit interact through the surface-exposed loop (residues 112-121 in blade 4) of one i/TIF34 molecule with β-sheet in blade 1 and loop in blade 2 of another i/TIF34 molecule, as depicted by the red arrow. Complex I denotes chains E and B, as named in the pdb file, and complex II – chains F and D.

**Figure S5.** Positional comparison of the i/TIF34 residues directly interacting with b/PRT1 with those identified in previous mutational studies by Asano and co-workers (10), which cluster on opposite sides of beta-propeller. Positions of eight lethal mutations are shown as red dots. The residues include Y18, C28, H51, D61, G257 (to D or S), G294 and D314. Ts′ changes at positions G311 and P247 are shown as blue dots. In the latter work, amino acids conferring Ts′ or Sgst′ mutations were identified in blades 6 (P247L and Q258R) and 7 (G311S). Direct comparison shown here clearly reveals that none of these residues form contacts to b/PRT1(654-700). Mutations P247L and G311S were proposed to affect mainly binding of g/TIF35, since overexpression of g/TIF35 could fully suppress the observed phenotypes (10). Notably, P247 is in close proximity to the extended C-terminal part of b/PRT1 (starting with residue 689) suggesting that both b/PRT1 and g/TIF35 might share the same binding surface on i/TIF34. In agreement with this proposal, the Ts′ mutation Q258R, which was only weakly suppressed by the high copy TIF35 plasmid and conferred no defects in binding to b/PRT1 or g/TIF35 (10), resides at the base of the flexible loop (residues 260-272) on the opposite site of the b/PRT1 binding site.

**Figure S6.** i/TIF34 residues 260-272 fold into a flexible loop. (A) The flexible loop within blade 6 (residues 260-272) displays well-defined electron density only in one
molecule of the asymmetric unit (complex II, chain D in the pdb file - shown with the corresponding 2mFo-DFc electron density map contoured at 1.0 sigma). The loop in another molecule (complex I, chain B) is not visible and therefore the residues have been omitted from the pdb file. The red arrow indicates the site of the lacking flexible loop in the complex I. (B) Side view showing the flexible loop (indicated by black bracket), which bears neutral-to-negative charge.

Figure S7. Gel shift analysis of i/TIF34 binding to b/PRT1 mutants. (A) b/PRT1(630-739) binds i/TIF34 with a $K_D$ of 0.26 μM±0.15, as determined by directly titrating 0.1 μM $[^{35}S]$-b/PRT1 with increasing amounts of i/TIF34 (0.1-25 μM) and calculated using DynaFit software (58,59). (B) Competition assays were used to test binding of b/PRT1 mutants. 0.2 μM $[^{35}S]$-b/PRT1(630-724) was mixed with 1 μM i/TIF34, and increasing amounts of unlabeled b/PRT1 peptides were added to the reaction mixture (see Supplementary Materials and Methods). Complexes were incubated at 30°C temperature in a final volume of 20 μl reaction mix containing 20 mM Hepes pH 7.5, 50 mM NaCl and 5% glycerol for 20 min and analyzed on 4-16% native polyacrylamide BisTris gels (Invitrogen). The b/PRT1(654-700) peptide and W674F mutant bind i/TIF34 with similar affinity as the b/PRT1(630-724). The increments of increase (the ratio of concentrations of unlabeled to labeled proteins) were as follows: panel b(630-724): 0.1, 0.5, 1, 10, 25, 50, 100; panel W674F: 1, 2, 10, 25, 50, 100; panel W674A: 1, 10, 25, 50, 100, 200; panel Y677A/R678A: 1, 10, 25, 50, 200; panel b(654-700): 1, 5, 50, 100, 200.

Figure S8. (A) The tif34-DD/KK mutation disrupts i/TIF34 binding to b/PRT1 while preserving its contact with g/TIF35 in vivo. WCEs were prepared from H450 (tif34Δ) bearing wt TIF34-HA and empty vector (lanes 1 – 4), wt TIF34-HA and 8x His-tagged hc TIF35 (lanes 5 – 8) and mutant tif34-DD/KK-HA and 8x His-tagged hc TIF35 (lanes 9 – 12) and analyzed as in Figure 5A - B. (In) lanes contained 5% of the input WCEs; (E1) lanes contained 10% of eluate from the resin; (E2) lanes contained 20% of eluate from the resin; and (FT) lanes contained 5% of the flow through. (B) The tif34-D207K and tif34-D224K mutations are redundant and have only mild effect on the MFC composition in vivo. WCEs were prepared from YAH12 (prt1Δ tif34Δ) bearing untagged PRT1 and wt TIF34 on sc plasmids (lanes 1-3), or from YAH11 (prt1Δ tif34Δ) bearing 8x His-tagged PRT1 and either wt TIF34 (lanes 4-6), mutant tif34-D207K (lanes 7-9) or mutant tif34-D224K (lanes 10-12), and analyzed as in Figure 5A - B. (In) lanes contained 5% of the input WCEs; (E) lanes contained 100% of eluate from the resin; and (FT) lanes contained 5% of the flow through. (C) The prt1-R678A and prt1-Y677A mutations are redundant and have only mild effect on the MFC composition in vivo. WCEs were prepared from YAH06 (prt1Δ) bearing untagged PRT1 (lanes 1 – 3), 8x His-tagged PRT1 (lanes 4 – 6) and two of its mutant derivatives (lanes 7 – 9 and 10 - 12) and analyzed as in Figure 5A - B. (In) lanes contained 5% of the input WCEs; (E) lanes contained 100% of eluate from the resin; and (FT) lanes contained 5% of the flow through. (D) The prt1-W674A mutation provokes severe leaky scanning defect. The YAH06 (prt1Δ) strains bearing either wt
*PRT1* or mutant *prt1-W674A* were transformed with the *GCN4-lacZ* reporter plasmids pM226 (i) and plig102-3 (ii), grown in SD medium at 34°C and analyzed as in Figure 6D.
## Supplementary Table S1. Data collection and refinement statistics (MIR)

|                          | D61 (native) | HgThioMer | Pt |
|--------------------------|--------------|-----------|----|
| **Data collection**      |              |           |    |
| Space group              | P3 1 2 1     | P3 1 2 1 | P3 1 2 1 |
| Cell dimensions          |              |           |    |
| $a$, $b$, $c$ (Å)        | 126.28 126.28 105.64 | 125.9 125.9 105.45 | 125.9 125.9 107.58 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90 90 120 | 90 90 120 | 90 90 120 |
| Resolution (Å)           | 54.7-2.2 (2.32-2.2) | 75.8-2.99 (3.15-2.99) | 54.5-3.8 (4-3.8) |
| $R_{merge}$ (%)          | 13.5 | 22.0 | 31.8 |
| $<\langle I\rangle>/<\sigma(I)>$ | 10.2 (2.2) | 9.3 (2.1) | 2.9 (1.1) |
| Completeness (%)         | 99.9 (100) | 97.5 (83.1) | 99.9 (100) |
| Multiplicity             | 7.4 (7.6) | 7.6 (7.3) | 3.8 (3.7) |
| Wavelength (Å)           | 1.00000 | 1.5418 | 1.5418 |
| **Phasing**              |              |           |    |
| Phasing power, acentric  |              | 0.98/0.21 | 0.25/0.05 |
| (Isomorphous/Anomalous)  |              |           |    |
| Mean Figure of merit,    |              | 0.16 (0.81) |    |
| acentric (inner resolution bin) | | | |
| $<\text{FoM}>$ after solvent flattening (53%) | 0.69 | | |
| **Refinement**           |              |           |    |
| No. reflections          | 47261 | | |
| $R_{work}$ / $R_{free}$ (%) | 19/24 | | |
| No. atoms                |              |           |    |
| Protein                  | 6145 | | |
| Water                    | 237 | | |
| $<B>$ factors (Å²)       |              |           |    |
| Protein                  | 37.6 | | |
| Water                    | 38.9 | | |
| R.m.s. deviations        |              |           |    |
| Bond lengths (Å)         | 0.022 | | |
| Bond angles (°)          | 1.867 | | |
| Ramachandran plot (%)d   |              |           |    |
| Favoured                 | 96.31 | | |
| Allowed                  | 3.43 | | |
| Outliers                 | 0.26 | | |

*a* Data were collected on a single crystal, and in the refinement, 5% of unique reflections were removed as a test set for $R_{free}$ calculation.

*b* Values in parentheses are for highest-resolution shell.

*c* $R_{merge} = \frac{\sum |I_{hl} - \langle I_{hl} \rangle|}{\sum \langle I_{hl} \rangle}$ where $I_{hl}$ is the $i^{th}$ measurement of reflection $h$.

*d* From MolProbity (29)
### Supplementary Table S2. The i/TIF34-b/PRT1 interaction interface

| b/PRT1   | Distance, Å | i/TIF34          |
|----------|-------------|------------------|
| **Contacts involving side chains**          |             |                  |
| F:Tyr677 (OH)<sup>a</sup>                  | 2.70        | D:Asp224 (OD1)<sup>a</sup> |
| F:Arg678 (NH1)                             | 3.49        | D:Thr209 (OG1)   |
| **Main-chain contacts**                     |             |                  |
| F:Phe694 (N)                               | 3.35        | D:Asp207 (OD2)   |
| F:Met689 (O)                               | 2.96        | D:Lys164 (NZ)    |
| **Salt bridges**                            |             |                  |
| F:Arg678 (NH1)                             | 2.99        | D:Asp207 (OD2)   |
| F:Arg678 (NH2)                             | 2.75        | D:Asp207 (OD2)   |

<sup>a</sup> F, D is the chain id. Contacts in another complex from the ASU, between chains E and B are the same with minor distance deviations. The interaction parameters were calculated using PISA tool (35).
### Table S3. Yeast strains used in this study

| Strain | Genotype                                                                 | Source or reference |
|--------|---------------------------------------------------------------------------|---------------------|
| YAH06  | MATa leu2-3,112 ura3-52 trp1A prt1::hisG GCN2 (hc PRT1 URA3)               | (12)                |
| AY134  | MATa leu2-3,112 ura3-52 trp1A prt1::hisG GCN2 (hc PRT1 LEU2)              | This study          |
| YAH11  | MATa leu2-3,112 ura3-52 trp1A prt1::hisG tif34Δ GCN2 (hc PRT1-His LEU2 and hc TIF34 URA3) | This study          |
| YAH12  | MATa leu2-3,112 ura3-52 trp1A prt1::hisG tif34Δ GCN2 (hc PRT1, URA3 and sc TIF34-HA TRP1) | This study          |
| H450   | MATa leu2-3,-112 ura3-52::GCN2 trp1Δ tif34Δ (hc TIF34 URA3)                | L. Cuchalová        |
| H420   | MATa leu2-3,-112 ura3-52 trp1 gcn2Δ tif34Δ (hc TIF34 URA3)                 | Klaus H. Nielsen    |
| H417   | MATa leu2-3,-112 ura3-52 trp1                                            | Klaus H. Nielsen    |
| H418   | MATa leu2-3,-112 ura3-52 trp1 gcn2Δ                                      | Klaus H. Nielsen    |
### Table S4. Plasmids used in this study

| Plasmid | Description | Source of reference |
|---------|-------------|---------------------|
| pRS-b/PRT1-HisXS | low copy PRT1 in LEU2 plasmid from pRS315 | (12) |
| pRS-b/PRT1-W674A-His | low copy PRT1 containing the W674A mutation in LEU2 plasmid from pRS315 | This study |
| pRS-b/PRT1-W674F-His | low copy PRT1 containing the W674F mutation in LEU2 plasmid from pRS315 | This study |
| pRS-b/PRT1-Y677A-His | low copy PRT1 containing the Y677A mutation in LEU2 plasmid from pRS315 | This study |
| pRS-b/PRT1-R678A-His | low copy PRT1 containing the R678A mutation in LEU2 plasmid from pRS315 | This study |
| pRS-b/PRT1-Y677A-R678A-His | low copy PRT1 containing Y677A and R678A mutations in LEU2 plasmid from pRS315 | This study |
| pRS-b/PRT1-Y677A-R678D-His | low copy PRT1 containing Y677A and R678D mutations in LEU2 plasmid from pRS315 | This study |
| YEp-TIF34-U | high copy TIF34 URA3 plasmid from YEplac195 | (10) |
| YCp-i/TIF34-HA | single copy wt TIF34-HA in LEU2 plasmid from YCplac111 | (10) |
| YCp-i/TIF34-D207K-HA | single copy wt TIF34-HA containing the D207K mutation in LEU2 plasmid from YCplac111 | This study |
| YCp-i/TIF34-D224K-HA | single copy wt TIF34-HA containing the D224K mutation in LEU2 plasmid from YCplac111 | This study |
| YCp-i/TIF34-D207K-D224K-HA | single copy wt TIF34-HA containing D207K and D224K mutations in LEU2 plasmid from YCplac111 | This study |
| YCp-i/TIF34-L222D-HA | single copy wt TIF34-HA containing the L222D mutation in LEU2 plasmid from YCplac111 | This study |
| YCp-i/TIF34-L222K-HA | single copy wt TIF34-HA containing the L222K mutation in LEU2 plasmid from YCplac111 | This study |
| YCp-i/TIF34-HA-W | single copy wt TIF34-HA in TRP1 plasmid from YCplac22 | This study |
| YCp-i/TIF34-D207K-D224K-HA-W | single copy wt TIF34-HA containing D207K and D224K mutations in TRP1 plasmid from YCplac22 | This study |
| YCp-i/TIF34-D224K-HA-W | single copy wt TIF34-HA containing D224K mutation in TRP1 plasmid from YCplac22 | This study |
| YEpplac195 | high copy URA3 plasmid | (64) |
| YEp-g/TIF35 | high copy wt TIF35 in URA3 plasmid from YEp352 | (10) |
| YCp-g/TIF35-His-screen | single copy wt TIF35-His in TRP1 plasmid from YCplac22 | (5) |
| YEp-g/TIF35-His | high copy wt TIF35-His in URA3 plasmid from YEpplac195 | This study |
| YEp-SUI1 | high copy SUI1 (eIF1) in URA3 plasmid from YEplac195 | (39) |
| pCFB130 | high copy sui1-His containing the 93-97 mutation in LEU2 plasmid form YEpplac181 | (47) |
| pICB02 | high copy sui1-His containing the D83G mutation in LEU2 plasmid form YEpplac181 | (47) |
| pICB04 | high copy sui1-His containing the Q84P mutation in LEU2 plasmid form YEpplac181 | (47) |
| pCFB134 | high copy sui1-His containing the G107R mutation in LEU2 plasmid form YEpplac181 | (47) |
| YEpl-SUI1-G107R | high copy sui1 containing the G107R mutation in URA3 plasmid from YEplac195 | This study |
| pGEX-5X-3 | cloning vector for GST fusions | (63) |
| plasmid | description |
|---------|-------------|
| pGEX-i/TIF34 | GST-i/TIF34 fusion plasmid from pGEX-5X-3 (10) |
| pGEX-g/TIF35 | GST-g/TIF35 fusion plasmid from pGEX-5X-3 (10) |
| pGEX-b/PRT1 | GST-b/PRT1 fusion plasmid from pGEX-5X-3 (13) |
| pGEX-RPS0A | GST-RPS0A fusion plasmid from pGEX-4T-1 This study |
| pT7-b/PRT1 | PRT1 ORF cloned under T7 promoter into pT7-7 (10) |
| pT7-b/PRT1-W674A | PRT1 ORF containing the W674A mutation cloned under T7 promoter into pT7-7 This study |
| pT7-b/PRT1-Y677A | PRT1 ORF containing the Y677A mutation cloned under T7 promoter into pT7-7 This study |
| pT7-b/PRT1-R678A | PRT1 ORF containing the R678A mutation cloned under T7 promoter into pT7-7 This study |
| pT7-i/TIF34 | TIF34 ORF cloned under T7 promoter into pT7-7 (10) |
| pT7-i/TIF34-D207K-D224K | TIF34 ORF containing D207K and D224K mutations cloned under T7 promoter into pT7-7 This study |
| pM226 | Derivative of pM199; ORF of uORF1 extends into the GCN4-lacZ coding region (61) |
| plig102-3 | Low copyURA3 vector with GCN4 leader point mutations containing UORF4 only at its original position in front of the GCN4-lacZ coding region (61) |
| Primer name         | Primer sequence (5’ to 3’)                                                                 |
|--------------------|---------------------------------------------------------------------------------------------|
| AH-PRT1-BamHI      | TCAC TTGGGATCCATCTGGTA                                                                      |
| AH-PRT1-NotI-R     | CGCGGTCGGCGGCGGCGGATCT                                                                     |
| AH-PRT1-W674A-R    | TGCTTGCTTCAATAATTTCACGTTG                                                                  |
| AH-PRT1-W674A      | CAACGTGAATTATGCAAGCAACCGAATTAGAGAAAATT                                                     |
| AH-PRT1-W674F-R    | GAATTCGCTTCAATAATTTCACGTTG                                                                  |
| AH-PRT1-W674F      | CAACGTGAATTATGCAAGCAACCGAATTAGAGAAAATT                                                     |
| AH-PRT1-YR-AA-R    | TTTTTCTACTTCCTTGCCAAATTCTTTTCTTGCTGTTGTCATTTGCAATAATAA                                    |
| AH-PRT1-YR-AD-R    | TTTTTCTACTTCCTTGCCAAATTCTTTTCTTGCTGTTGTCATTTGCAATAATAA                                    |
| AH-PRT1-Y677A-R    | TTCAATTCTTCGCAATTTTTTCTTTTGCTGTTGTCATTTGCAATAATAA                                         |
| AH-PRT1-R678A-R    | TTCAATTCTTCGCAATTTTTTCTTTTGCTGTTGTCATTTGCAATAATAA                                         |
| y3iKpnI           | TACCTTGAGTCGACATGACGTACCACCAGTACC                                                      |
| BmgBlr            | CGCTCTTCCAGCTCGTATTTGAAAT                                                                |
| D207K             | GTGACATGCAATTCTCTCTCTGTTACATACTTTATTACATCCAGAGATA                                         |
| D207Kr            | AGGAGAAATTGCTGACATCAGTGGA                                                               |
| D224K             | ATACTAATCTCGTCTGTTGCAAGGATCGACTCTACAAGTCTTGAAGAATACG                                      |
| D224Kr            | GACCTAGAAGGATTGTACATCTTGGA                                                              |
| LC3iL222D         | ACATCATCCAGAGATATACTACTCGTGACGTAGTGATCGACTCTACAGAAGATCTT                                 |
| LC3iLr            | GAACGAGTTAGTACTCGTCTGCAGT                                                               |
| LC3iL222K         | ACATCATCCAGAGATATACTACTCGTGACGTAGTGATCGACTCTACAGAAGATCTT                                 |
| O1-BamHI          | AATAGGATGCCATATCTGCTGCTTTACACGTACTTTTGA                                                |
| BSRPS0Aexon1-r    | TGAAAACATACGGTTCTTGGTGAAACCTGACCTTCAAGATAGTGCAAGAC                                       |
| BSRPS0Aexon2      | CAAGAACCAGTATGTGTTTACACCGCAAAGACC                                                      |
| O2-KpnI-XhoI-r    | TTTCGAGATGGTACCTTACACGCTGACGCAGTTGATGCATTTT                                            |
