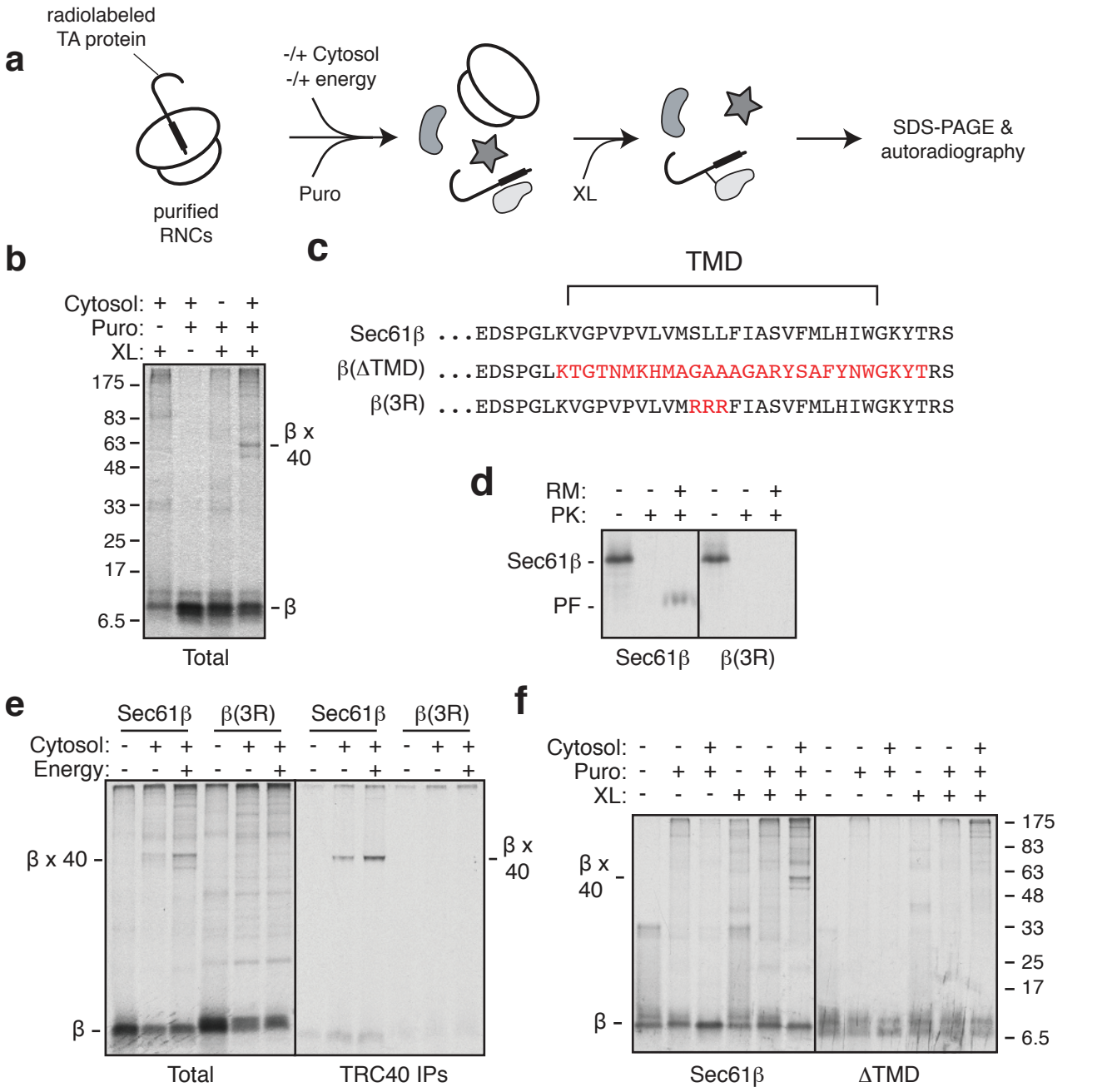


Fig. S1. In vitro reconstitution and characterization of TA protein capture by TRC40. (**a**) Experimental design. Sucrose-gradient purified ribosome-nascent chains (RNCs) of the model TA protein Sec61β are released with puromycin in the presence or absence of cytosolic fractions and an energy regenerating system. The resulting interactions are detected by chemical crosslinking, SDS-PAGE, autoradiography., and/or immunoprecipitation. (**b**) Purified Sec61β RNCs were treated as indicated and the crosslinks analyzed by SDS-PAGE. The positions of the non-crosslinked Sec61β and the major adduct with TRC40 are indicated. Note that the crosslinked adduct with TRC40 is only observed upon release with puromycin. (**c**) Sequences of the TMD region of wild type Sec61β and the indicated mutants. Differences from wild type are in red. (**d**) Valiation that the Sec61β(3R) mutant is not competent for insertion. Protease-protection assays for insertion into ER-derived microsomes was performed as before [Stefanovic and Hegde (2007) *Cell* **128**:1147-59]. The protected fragment (PF) corresponding to the inserted TMD is indicated. (**e**) RNCs of wild type Sec61β or Sec61β(3R) were analyzed for capture by TRC40 in the absence or presence of an energy regenerating system. The total products and TRC40 immunoprecipitates (IPs) are shown. Note that the insertion-deficient 3R mutant is not captured by TRC40. (**f**) Sec61β containing or lacking an intact TMD was analyzed by the RNC release assay as in panel b. Replacement of the TMD with an irrelevant hydrophilic sequence completely abrogates interactions with TRC40. Identical results were obtained when the TMD was simply deleted (data not shown)

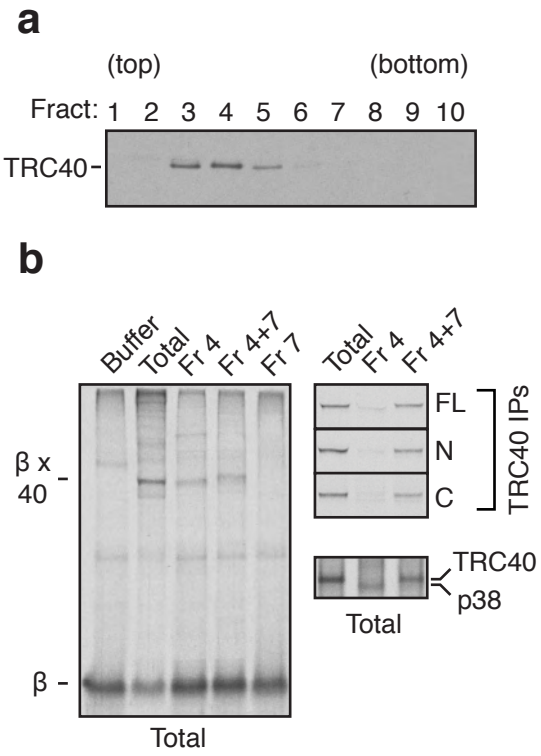


Fig. S2. An additional factor is needed for substrate capture by TRC40.(**a**) Cytosol was fractionated on a 5-25% sucrose gradient and the individual gradient fractions analyzed by immunoblotting for TRC40. The peak of TRC40 is in fraction 4; note that fraction 7 contains little if any TRC40. (**b**) Substrate capture assay in the indicated sucrose gradient fractions were either analyzed directly (left) or immunoprecipitated with anti-TRC40 antibodies (right). An approximately 40 kD crosslinking partner is seen in three of the lanes. However, the primary crosslink in Fraction 4 cannot be immunoprecipitated by TRC40 antibodies raised against the N-terminus, C-terminus, or full length (FL) protein (uper right panel). On lower percentage gels, this crosslink (p38) can be resolved from crosslinks to TRC40 (lower right panel). This p38 crosslink is lost and TRC40 regained when a mixture of fractions 4 and 7 are used. Since fraction 7 does not itself contain TRC40, this result suggests that fraction 7 contains a factor that facilitates capture of substrate by TRC40. We believe p38 is another cytosolic protein capable of binding hydrophobic sequences since its interaction was TMD-dependent.

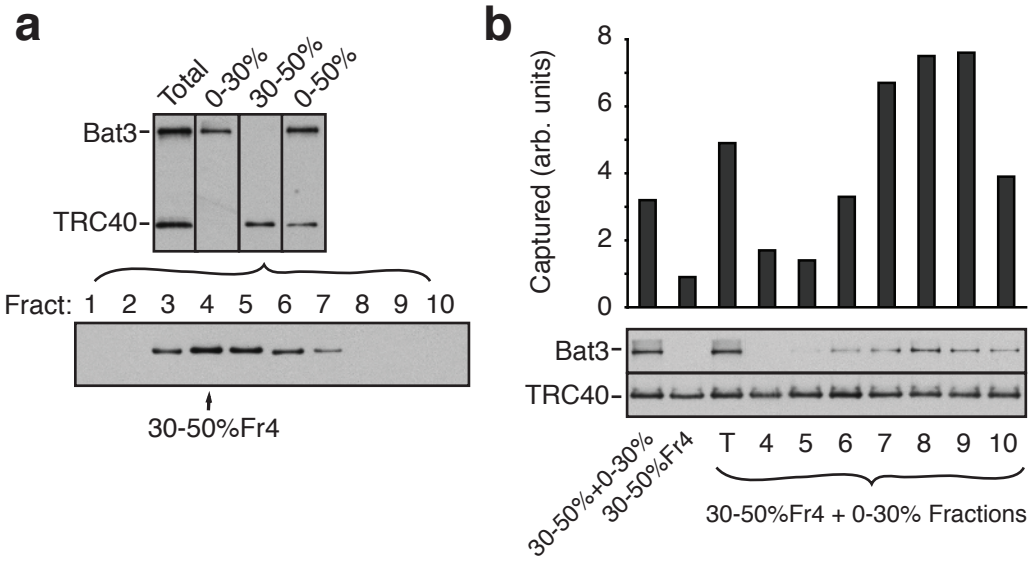


Fig. S3. Properties of the capture-stimulating activity and co-fractionation with Bat3. (**a**) Ammonium sulfate (AS) fractionation and analysis of the fractions by immunoblotting for TRC40 and Bat3. Bat3 is efficiently precipitated by 30% AS, while TRC40 requires 50%. The 30-50% AS cut was further separated on a sucrose gradient, and the peak TRC40-containing fraction (fraction 4) was tested in panel b for its ability to capture Sec61β in the RNC release assay as in Sup. Fig. S1. The 0-30% AS cut was also separated on a sucrose gradient, and the individual fractions tested for their ability to stimulate TRC40 capture of Sec61β. (**b**) Capture assays of Sec61β RNCs in the indicated fractions. 'T' is the total 0-30% ammonium sulfate fraction, while individual numbers indicate fractions from a sucrose gradient separation of the 0-30% sample. The products of the assay were immunoprecipitated with anti-TRC40 and quantified to assess capture activity. The same samples were also analyzed by immunoblotting for Bat3 and TRC40. Note that maximal capture by TRC40 occurs in fractions containing Bat3. The somewhat lower activity in the crude ammonium sulfate precipitate samples is likely due to inhibition by residual ammonium sulfate. Additional fractionation steps by ion exchange also showed a correlation between Bat3 and capture-stimulating activity (data not shown).

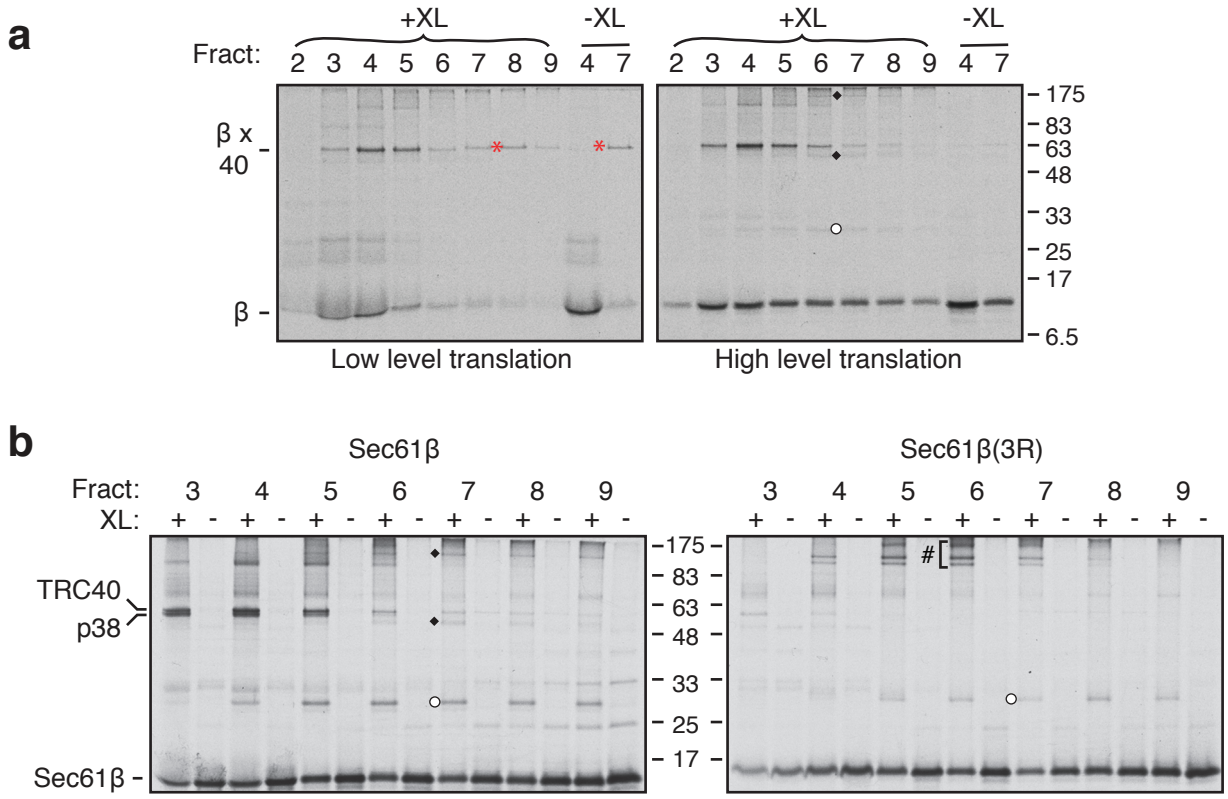


Fig. S4. Crosslinking reveals candidate proteins for the capture-stimulating factor. (**a**) Full length Sec61β was translated in vitro at low or high levels by adjusting the concentration of transcript. Preliminary experiments were used to identify maximal translation conditions (high level), as well as transcript levels needed to produce ~5-fold less product (low level). After translation, the samples were separated on a 5-25% sucrose gradient and each fraction subjected to crosslinking analysis. Aliquots of fractions 4 and 7 in the absence of crosslinker are also shown. Loading of the gel was adjusted such that equal amounts of [35S]-labeled Sec61β were analyzed. The major crosslink (to TRC40) in fractions 3-6 is indicated. (**b**) Sec61β or Sec61β(3R) was translated under 'high level' translation conditions and analyzed for crosslinking to identify interacting partners that are selective to an insertion-competent TMD. The samples without and with crosslinker are shown for fractions 3 to 9. The diamonds and open circles are the same products indicated in panel a. The '#' symbol indicates crosslinks that are unique to the 3R construct. Note that upon saturation of the TRC40 system, crosslinks to p38 can observed.

Two observations are noteworthy. First, Sec61β migrates more heterogeneously at high levels compared to low levels, with a greater proportion of product seen in the later sucrose gradient fractions (compare the relative amounts of Sec61β in fractions 4 and 7 of the non-crosslinked samples of each translation reaction). Second, this population of Sec61β seen preferentially in the high-level translations is observed to form crosslinks to partners of ~15, 35, and 120 kD. Of these, the 15 kD crosslinking partner (open circle) does not appear to be strictly TMD-dependent (see panel b) and is not Ubl4A, while the ~35 and ~120 kD crosslinking partners (diamonds) proved to be TRC35 and Bat3 in subsequent immunoprecipitation studies.

We estimate by immunoblotting relative to standards that under high-level translation conditions, the amount of Sec61β translated in vitro is ~ 1 ng/ul, which would exceed by ~2-4-fold the relative molar amount of endogenous TRC40 (i.e., it can be saturated under these conditions).

The band marked by the red asterisk is a reticulocyte protein that becomes non-specifically labeled, and is only observed when samples are over-loaded. Note its presence even in the absence of crosslinker. It can also be seen faintly in the high-level translation samples.

Distortion of the Sec61β band in the low level translation gel is due to co-migrating hemoglobin in the reticulocyte lysate. This distortion is not seen in the high level translation because ~5-fold less translation reaction was loaded.

a

MEPNDSTSTAVEEPDSLEVLVKTLDSQTRTFIVGAQMNVKEFKEHIAASVSIPSEKQRLIYQGRVLQDDKK
LQEYNVGGKVIHLVERAPPQTHLPSGASSGTGSASATHGGGSPPGTRGPGASVHDRNANSYVMVGTFNLPS
DGSAVDVHINMEQAPIQSEPRVRLVMAQHMIRDIQTLLSRMETLPYLQCRGGPQPQHSQPPPQPPAVTPEP
VALSSQTSEPVESEAPPREPMEAEEVEERAPAQNPELTPGPAPAGPTPAPETNAPNHPSPAEYVEVLQELQ
RLESRLQPFLQRYYEVLGAAATTDYNNNHEGREEDQRLINLVGESLRLLGNTFVALSDLRCNLACTPPRHL
HVVRPMSHYTTPMVLQQAAIPIQINVGTTVTMTGNGTRPPPTPNAEAPPPGPGQASSVAPSSTNVESSAEG
APPPGPAPPPATSHPRVIRISHQSVEPVVMMHMNIQDSGTQPGGVPSAPTGPLGPPGHGQTLGQQVPGFPT
APTRVVIARPTPPQARPSHPGGPPVSGTLQGAGLGTNASLAQMVSGLVGQLLMQPVLVAQGTPGMAPPPAP
ATASASAGTTNTATTAGPAPGGPAQPPPTPQPSMADLQFSQLLGNLLGPAGPGAGGSGVASPTITVAMPGV
PAFLQGMTDFLQATQTAPPPPPPPPPPPPAPEQQTMPPPGSPSGGAGSPGGLGLESLSPEFFTSVVQGVLS
SLLGSLGARAGSSESIAAFIQRLSGSSNIFEPGADGALGFFGALLSLLCQNFSMVDVVMLLHGHFQPLQRL
QPQLRSFFHQHYLGGQEPTPSNIRMATHTLITGLEEYVRESFSLVQVQPGVDIIRTNLEFLQEQFNSIAAH
VLHCTDSGFGARLLELCNQGLFECLALNLHCLGGQQMELAAVINGRIRRMSRGVNPSLVSWLTTMMGLRLQ
VVLEHMPVGPDAILRYVRRVGDPPQPLPEEPMEVQGAERASPEPQRENASPAPGTTAEEAMSRGPPPAPEG
GSRDEQDGASAETEPWAAAVPPEWVPIIQQDIQSQRKVKPQPPLSDAYLSGMPAKRRKTMQGEGPQLLLSE
AVSRAAKAAGARPLTSPESLSRDLEAPEVQESYRQQLRSDIQKRLQEDPNYSPQRFPNAQRAFADDP

b

MAAAAAMAEQESARNGGRNRGGVQRVEGKLRASVEKGDYYEAHQMYRTLFFRYMSQSKHTEARELMYSGAL
LFFSHGQQNSAADLSMLVLESLEKAEVEVADELLENLAKVFSLMDPNSPERVTFVSRALKWSSGGSGKLGH
PRLHQLLALTLWKEQNYCESRYHFLHSADGEGCANMLVEYSTSRGFRSEVDMFVAQAVLQFLCLKNKSSAS
VVFTTYTYQKHPSIEDGPPFVEPLLNFIWFLLLAVDGGKLTVFTVLCEQYQPSLRRDPMYNEYLDRIGQLFF
GVPPKQTSSYGGLLGNLLTSLMGSSEQEDGEESPSDGSPIELD

c

MQLTVKALQGRECSLQVPEDELVSTLKQLVSEKLNVPVRQQRLLFKGKALADGKRLSDYSIGPNSKLNLVV
KPLEKVLLEEGEAQRLADSPPPQVWQLISKVLARHFSAADASRVLEQLQRDYERSLSRLTLDDIERLASRF
LHPEVTETMEKGFSK

Red = antigen for antibody
Yellow = Peptide identified by MS
Underlined = conserved domains

Supplementary Fig. S5. Sequences of human Bat3 (**a**), TRC35 (**b**), and Ubl4A (**c**) annotated to indicate the peptides identified by MS/MS (yellow), regions used for antibody production (red), and recognizable domains (underlined). In Bat3, the N-terminal region contains a ubiquitin-like (Ubl) domain, and the C-terminal region contains a putative BAG domain. The N-terminal region of Ubl4A contains a Ubl domain.

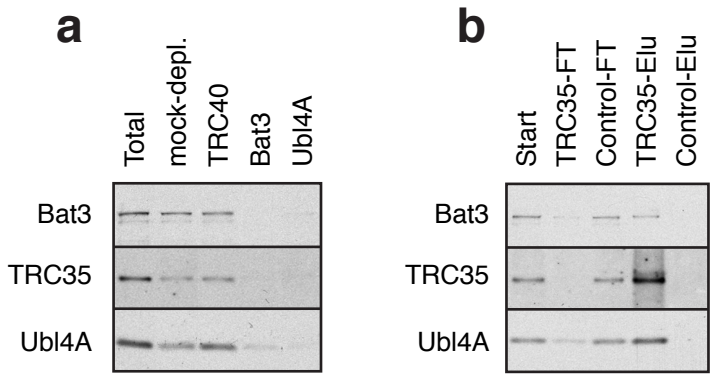
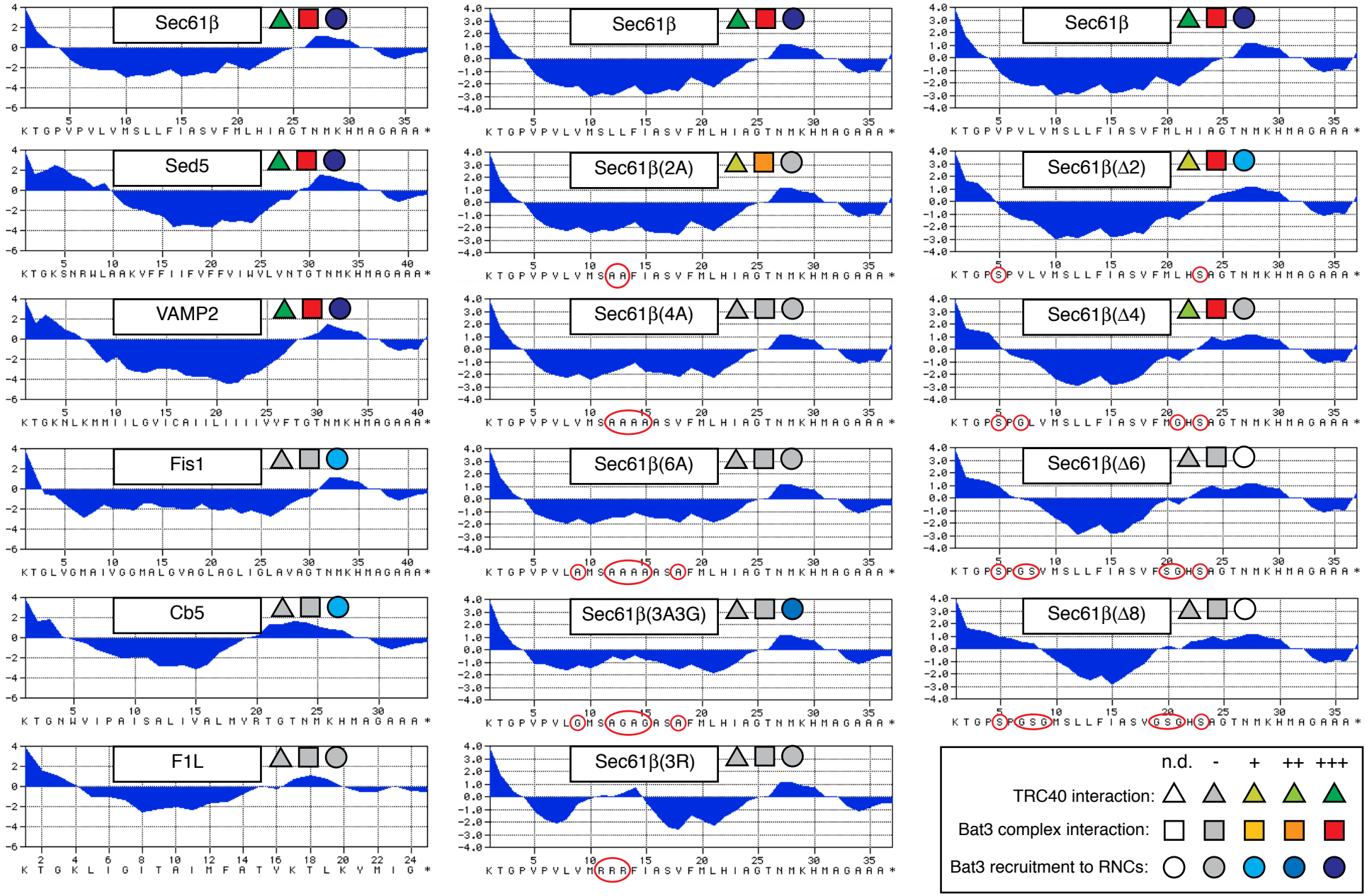
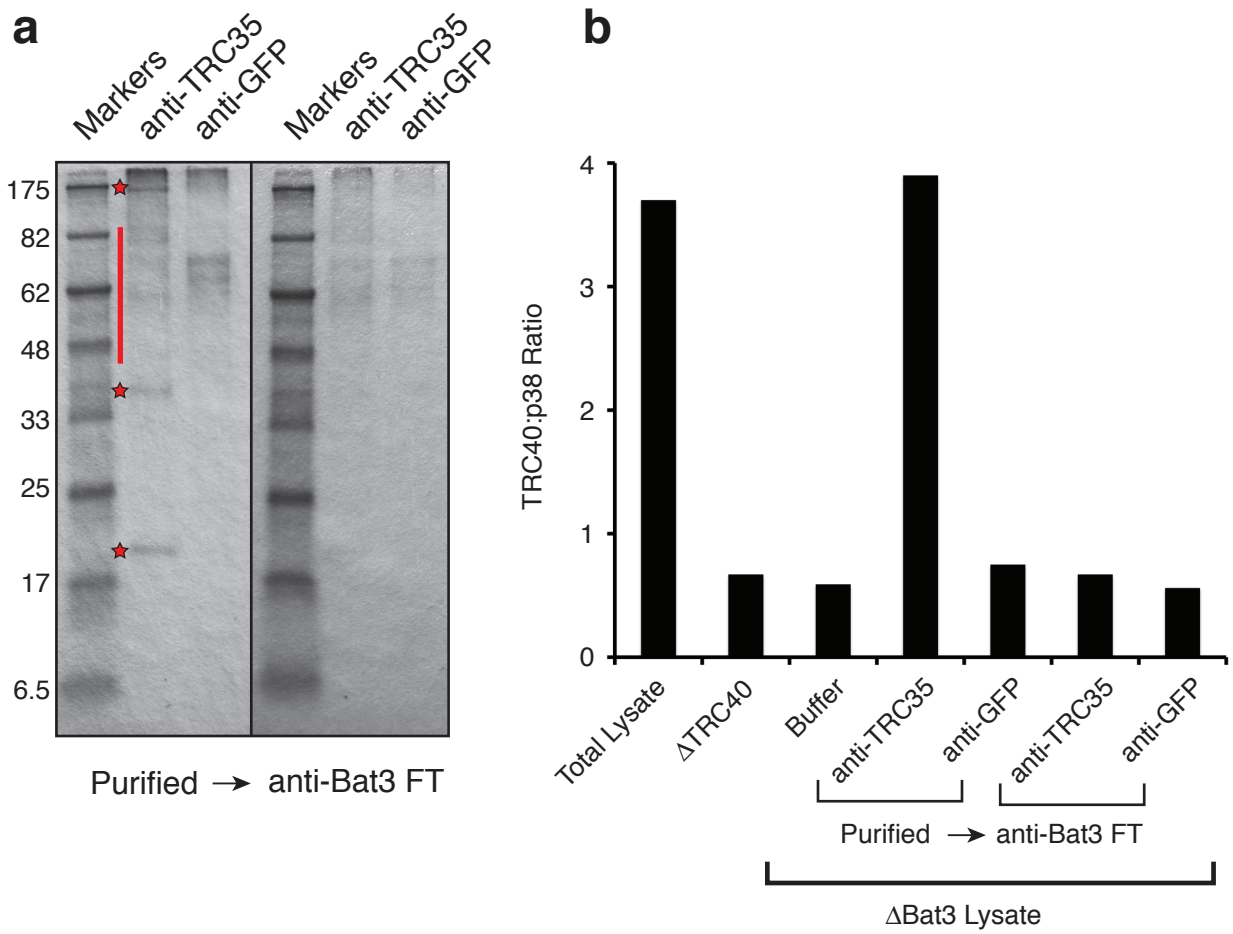


Fig. S6. Characterization of Bat3 complex interactions. (**a**) Depletion of Bat3 or Ubl4A co-depletes the other complex components. A reticulocyte cytosolic fraction (total) was passed over affinity columns containing control antibodies (to GFP; 'mock'), anti-TRC40, anti-Bat3, or anti-Ubl4A. The flow-through fractions from each column were analyzed by immunoblotting. We estimate that depletion of Bat3 results in ~70-80% depletion of Ubl4A and TRC35, with no effect on TRC40 (see Fig. 4A). (**b**) Co-purification of Bat3 and Ubl4A byTRC35 affinity chromatography. Reticulocyte lysate proteins eluted from a phenyl-sepharose column (start) were passed over either an anti-TRC35 or anti-GFP column (control). After washing, the columns were eluted with the immunizing TRC35 peptide. Aliquots of the flow-through and elution fractions were analyzed by immunoblotting against the Bat3 complex components. TRC40 was not detectable in the purified Bat3 preparations (data not shown).



Sup. Fig. S7 - Sequences and hydrophobicity of various TA protein constructs. The last ~40 residues of the indicated TA protein constructs used in this study were analyzed for hydrophobicity using the Kyte-Doolittle scale with window size 7. The left column shows native TMDs, and the other two columns show mutants of the Sec61β TMD (sequence changes indicated by red circles). Wild type Sec61β is shown at the top of each column for comparison. All constructs except F1L contain a C-terminal 3F4 epitope tag (TNMKHMAGAAA). All constructs are in the identical Sec61β context, with the only differences being the TMD. The functional properties of each construct are summarized using the color-coded symbols. A legend to these symbols is at lower left (n.d.= not determined).



Sup. Fig. S8 - Characterization of immunoaffinity purified Bat3 complex. (**a**) Native Bat3 complex purified by affinity chromatography using an anti-TRC35 antibody column (or a parallel anti-GFP control column), followed by concentration and detergent removal by cation exchange, was analyzed by SDS-PAGE and coomassie blue staining (left panel). The red stars indicate the bands corresponding to Bat3, TRC35, and Ubl4A. The red line indicates several apparent contaminants, primarily various IgG species, that leach from the affinity resin. To confirm this conclusion, these purified samples were passed over an anti-Bat3 antibody column, and the flow-thru fraction was analyzed in the right panel. Note that only the three primary bands of the Bat3 complex are selectively and quantitatively removed, while all of the contaminants remain. This indicates two things. First, that only the three proteins indicated are in a stable complex that can be isolated using one antibody (against TRC35) and depleted using another (against Bat3). Second, the other bands observed in the sample are recovered independently of an association with the Bat3 complex. (**b**) The samples from panel **a** were assayed for their ability to stimulate substrate capture by TRC40 in a lysate depleted of the Bat3 complex (by an anti-Bat3 resin). As controls, total lysate and TRC40-depleted lysate were analyzed in parallel. Plotted is the ratio of crosslinks to TRC40 versus p38. Note that complementation of the Bat3 depleted lysate with the eluate from the TRC35 affinity resin restored activity, while the control resin eluate had no activity. Importantly, passing the TRC35 eluate over the Bat3 resin completely depleted activity, demonstrating that the remaining contaminants have no activity in facilitating substrate capture by TRC40.

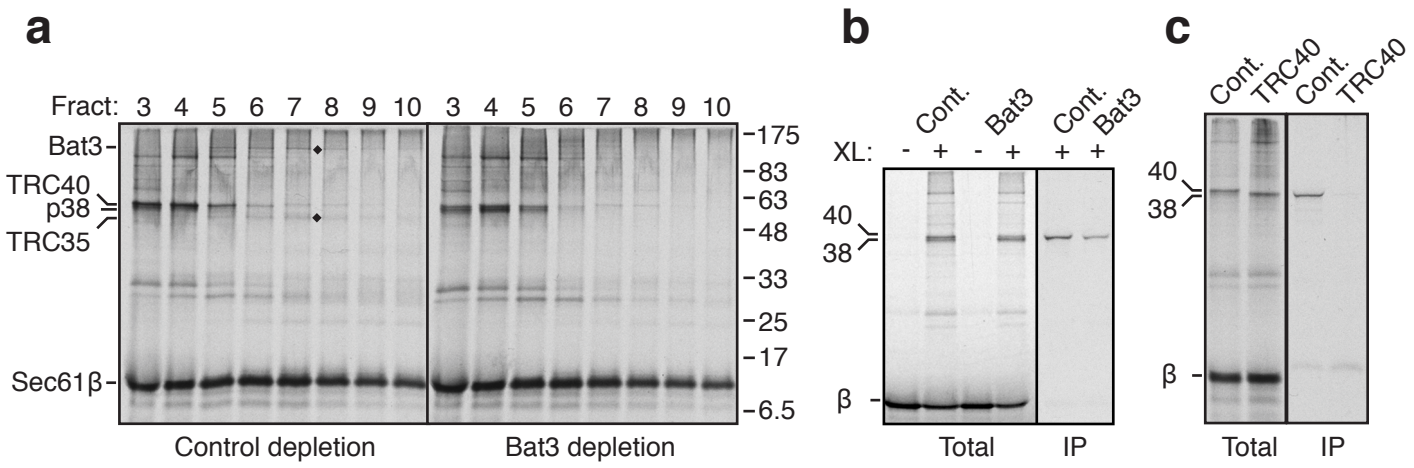
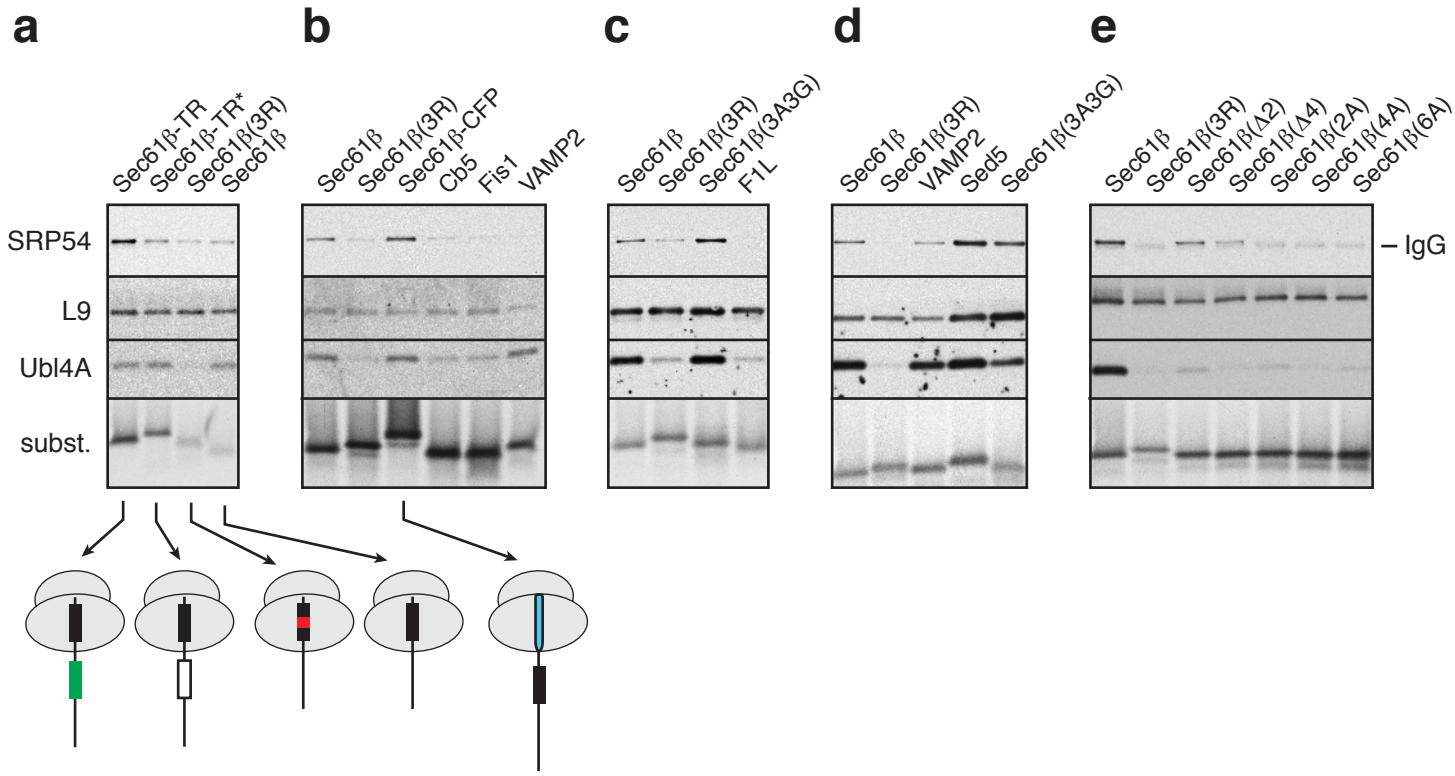


Fig. S9. Effect of Bat3 depletion on TA protein interactions. (**a**) Reticulocyte lysate translation extracts were immunodepleted using an anti-Bat3 or control antibody column and used for translation reactions of full length Sec61β. After translation, the samples were separated by 5-25% sucrose gradients and analyzed by crosslinking of individual fractions. The diamonds indicate crosslinks to Bat3 and TRC35, neither of which are observed in the Bat3-depleted translations. Crosslinks to TRC40 are observed in both translation reactions, but are diminished at the expense of crosslinks to p38 in the Bat3-depleted samples. This is seen more clearly in panel **b**, where fraction 4 (without and with crosslinker) is analyzed ('total' lanes). Immunoprecipitation with anti-TRC40 ('IP' lanes) and quantification by phosphorimaging verified that capture was reduced by ~60%. Blots of the depleted lysates verified that this reduction in capture was not due to any differences in TRC40 levels, which were identical between the two samples (data not shown). (**c**) Translation of Sec61β in lysates immunodepleted of TRC40 and analyzed by crosslinking as in panel **a** shows that TRC40 crosslinks are lost at the expense of p38 crosslinks. Shown are the crosslinking products from fraction 4, and the corresponding TRC40 immunoprecipitates (IP).



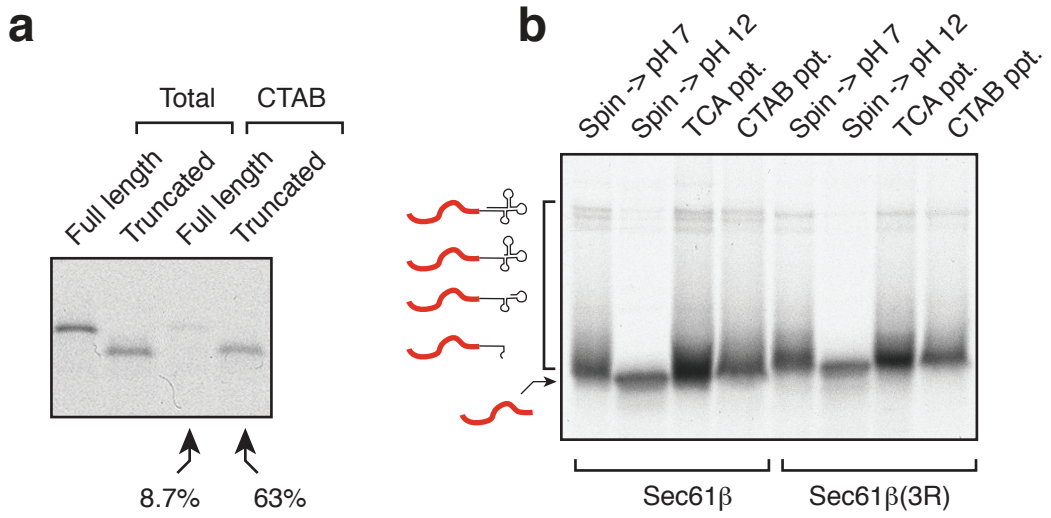
Sup. Fig. S10. Analysis of SRP and Bat3 complex recruitment to various RNCs. Panels **a** through **e** show different experiments where RNCs of the indicated substrates were immunoaffinity purified using anti-Sec61β and analyzed by immunoblotting against Ubl4A, SRP54, and ribosomal protein L9. The radiolabeled substrate ('subst.') was visualized by autoradiography. The experiment in panel **e** used magnetic beads containing antibodies that were not crosslinked to the bead, and hence, IgG is seen on the blot close to the SRP54 band.

Shown below some of the samples are schematic diagrams of the RNCs. The black rectangle represents the Sec61β TMD. The green rectangle represents the TMD from transferrin receptor (TR). The white rectangle represents an irrelevant hydrophilic sequence (encoded by the reverse sequence for TR). The black rectangle with red box represents the 3R mutant TMD of Sec61β. The cyan oval is hydrophilic sequence from CFP. The ribosomal tunnel shields ~30-40 residues, and the diagrams indicate this feature by illustrating which portions of the nascent chain are within or outside the ribosome. All other substrates that do not have an accompanying diagram contain the TMD (or mutant TMD) inside the ribosome.

The sequences of the various TMD regions and their accompanying hydrophobicity profiles are shown in Sup. Fig. S7. The sources of the native TMDs that were analyzed are as follows: VAMP2 was from rat; Sed5 was from S. cerevisiae; Fis1 was from human; F1L was from a vaccinia virus mitochondrially-targeted TA protein; Cb5 was from rabbit. The mutants either shorten the hydrophobic domain (i.e., Δ2 and Δ4) or reduce hydrophobicity while maintaining length (i.e., 2A, 4A, 6A, 3A3G).

The following observations are noteworthy. (1) Positioning a TMD outside the ribosome (Sec61β-TR or Sec61β-CFP) increases recruitment of SRP ~3-fold above that seen when a TMD is only inside the ribosome (e.g., Sec61β). (2) Increased SRP recruitment does not diminish the amount of Bat3 complex. (3) Recruitment of both SRP and Bat3 complex is sensitive to hydrophobicity, and generally follows a similar trend.

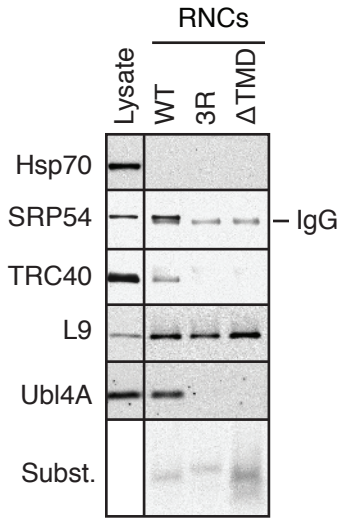
Note that Sec61β and Sec61β(3R) were included in all experiments as controls, and selected other constructs are represented in more than one experiment above. SRP54 is recovered to variable extents in the TMD-disrupted constructs such as the 3R mutant. This may be because of its substrate-independent association with ribosomes. Thus, the extent of washing or time taken during the isolation may influence its recovery in these samples.



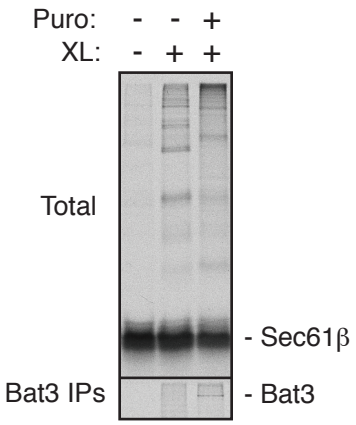
Sup. Fig. S11. Characterization of affinity purified RNCs for tRNA-association. (**a**) Selective precipitation of tRNA-associated, but not terminated, polypeptides using CTAB. Transcript coding for full length Sec61β or a truncated version lacking a stop codon was translated in vitro for 15 min at 32°C and analyzed directly ('Total') or subjected to precipitation with CTAB ('CTAB'). Equivalent amounts of sample were analyzed. All samples were treated with pH 12 buffer just before SDS-PAGE to hydrolyze any tRNA. The precent of translation product that is precipitated by CTAB was quantified by phosphorimaging and is shown below the respective lanes. Note that the small amount of CTAB precipitated full length protein was not background, as it was verified in later experiments to be lost upon puromycin treatment, and represents bona fide tRNA-associated product resulting from delayed termination (see Fig. 6). Thus, as characterized in earlier studies, CTAB can be used as a highly selective method to precipitate tRNA-associated polypeptides. (**b**) Immunoaffinity purified RNCs prepared as in Fig. 5B were divided into four equal aliquots and subjected to the following treatments: (1) Sedimentation to pellet ribosomes, followed by denaturation in pH 7 sample buffer. (2) Sedimentation followed by denaturation in pH 12 sample buffer. (3) Precipitation with trichloroacetic acid (TCA) to recover all products. (4) Precipitation with CTAB to recover tRNA-associated products.

The samples were then analyzed by SDS-PAGE on Tris-tricine gels. Because these gels are run in alkaline conditions (pH 8.5), tRNA is partially hydrolyzed during electrophoresis, resulting in a heterogeneous smear. This is collapsed to a single band upon alkaline treatment (pH 12 samples) or RNase digestion (data not shown). Note that the majority of recovered polypeptides are tRNA associated as judged by either their sensitivity to alkaline treatment or precipitation by CTAB.

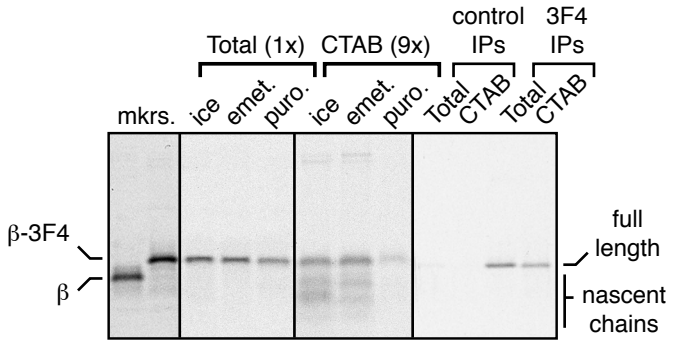
Furthermore, it is worth noting that analysis of RNCs after each step in the purification shows no reduction in the amount of tRNA-associated products recovered (data not shown). In fact, the tRNA-associated products are slightly enriched as the spontaneously hydrolyzed products are removed (especially in the gel filtration step).



Sup. Fig. S12. Magnetic bead isolation of TA protein RNCs. An experiment identical to that shown in Fig. 3B was performed using magnetic beads for the immunoaffinity isolation step. After RNCs were produced by in vitro translation and stabilized by emetine, they were enriched by gel filtration on Sephacryl S-300 resin. The void fraction (containing the RNCs) was incubated with magnetic protein A beads pre-bound with anti-Sec61β antibodies. After binding, the beads were washed multiple times using a magnet-based 'pull-up' strategy, and eluted by incubation with immunizing Sec61β peptide. The purified RNCs were analyzed by immunoblotting against the indicated antigens, For comparison, 0.5 ul of translation extract was analyzed in parallel (and shown from the same exposure as for the RNCs). Because the antibodies used for affinity purification was not immobilized on the magnetic beads, they can be observed just below the SRP54 band. Note that SRP54 and Bat3 complex were recovered in a TMD-dependent manner at high levels relative to their abundance in total lysate. By contrast, Hsp70 is not recovered at all, and TRC40 is found only a low levels, presumably due to its association with Bat3 complex.

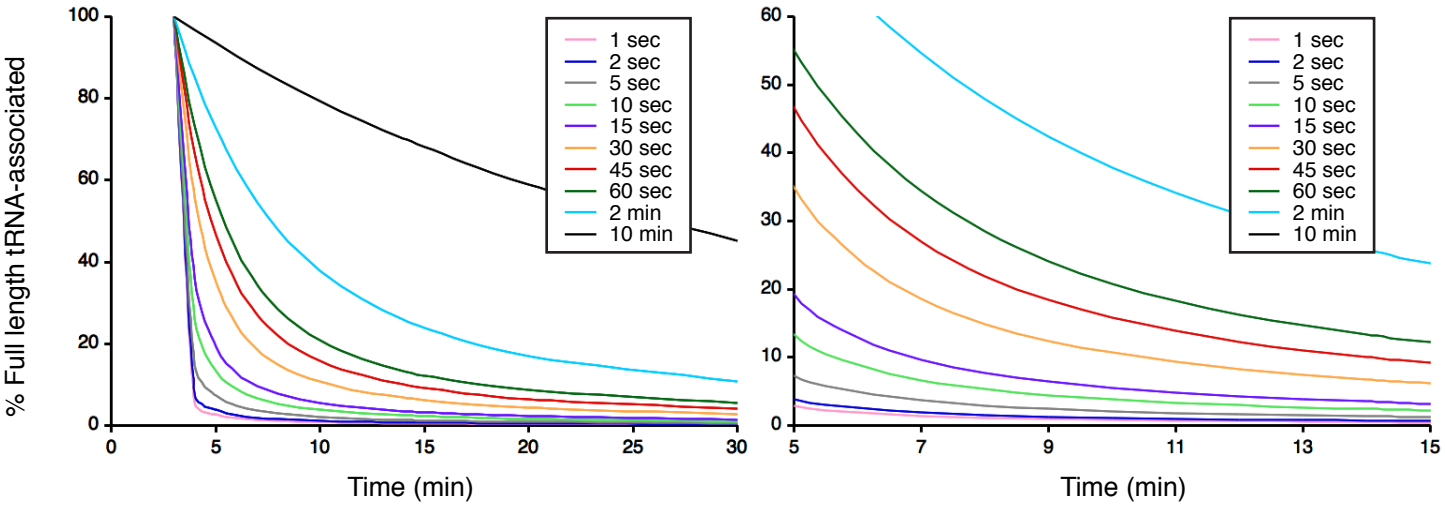


Sup. Fig. S13. Bat3 interacts with substrates post-translationally. A cytosolic lysate was fractionated by sucrose gradient sedimentation and a fraction containing Bat3 complex, but not TRC40, was isolated. This Bat3-containing fraction was mixed with Sec61β-RNCs and incubated with or without puromycin as indicated. The samples were then subjected to BMH crosslinking as indicated, and analyzed directly or after immunoprecipitation with anti-Bat3 antibodies. Note that Bat3 crosslinking to substrate was only observed after puromycin release, suggesting that its interaction is primarily post-translational.



Sup. Fig. S14. Analysis tRNA-associated nascent chains by CTAB precipitation. Sec61β up to and including the TMD, and Sec61β-3F4 (containing the 12-residue 3F4 epitope tag after the TMD), were synthesized in vitro and run as molecular weight markers (first two lanes). Sec61β-3F4 was synthesized for 10 min and either placed on ice, treated with 10 uM emetine for 10 min at 32°C, or treated with 1 mM puromycin for 10 min at 32°C. All samples were then analyzed directly ('Total'), after CTAB precipitation (9-fold more was analyzed), or after immunoprecipitation with 3F4 (or control) antibodies.

Nascent chains available for Bat3 complex recruitment would have synthesized the TMD (as judged by near full-length molecular weight), but still contain a covalent tRNA (and hence, be precipitated by CTAB), indicating that termination had not occurred. The above results illustrate the following. First, Sec61β containing a 12-residue 3F4 epitope tag following the TMD could be resolved easily from nascent chains that had not yet synthesized this epitope (but had made the TMD) (lanes 1 and 2). Second, CTAB precipitated products from a brief (10 min) translation reaction of Sec61β–3F4 showed a range of molecular weights enriched in non-full length polypeptpides representing partially synthesized nascent chains. Importantly, full length (or nearly so) polypeptides were clearly also precipitated, as verified by immunoprecipitation using 3F4 antibodies. Third, all of these products were markedly reduced if the translation reaction was first treated with puromycin, illustrating that they were functional translation intermediates. Conversely, treatment with emetine, a translation elongation inhibitor, stabilized these products. Thus, by combining CTAB precipitation with high resolution gels, we are able to identify near full length Sec61β-3F4 polypeptides that represent RNCs containing a TMD in the ribosomal tunnel. These nascent chains are precisely those which are able to recruit Bat3 complex, and what was quantified for the time course experiment in Fig. 4.



Sup. Fig. S15. Modeling of tRNA association for various translation termination rates. The expected amount of full length tRNA-associated product at various time points was calculated for various termination rates ranging from a $t_{1/2}$ of 1 sec to 10 min. The following parameters were used: (1) The first completed substrates appear at three minutes (validated by experiment). (2) Translation product accumulation is linear (validated to be true for 15 min in our experiments). (3) Termination follows a simple exponentional decay (defined by a $t_{1/2}$). Using these parameters, 100% of full length substrate will be tRNA-associated at 3 min. Before that, all products are tRNA-associated, but none are full length. After 3 min, more substrate continues to be produced, and existing full length tRNA-associated substrate terminates. Plotted on the graph is the proportion of total full length polypeptide (released and tRNA-associated) that is expected to be tRNA associated at any given time (and hence, should be precipitated with CTAB). The two graphs are the same, with the right graph showing only time points from 5 to 15 min, where reliable experimental data can be collected.

Note that although the experimental signal to noise is very low at the earliest time points (less than 5 min), we have indeed observed tRNA-associations approaching 100%, as expected from the predictions. However, the most reliable data is found after 5 min, where the amount of substrate is well above background. Hence, the experimental data in Fig. 4 was from 5 to 15 min, and was compared to the results shown in the right graph above.