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## Supplemental Information

# Identification of Oxa1 Homologs Operating in the Eukaryotic Endoplasmic Reticulum

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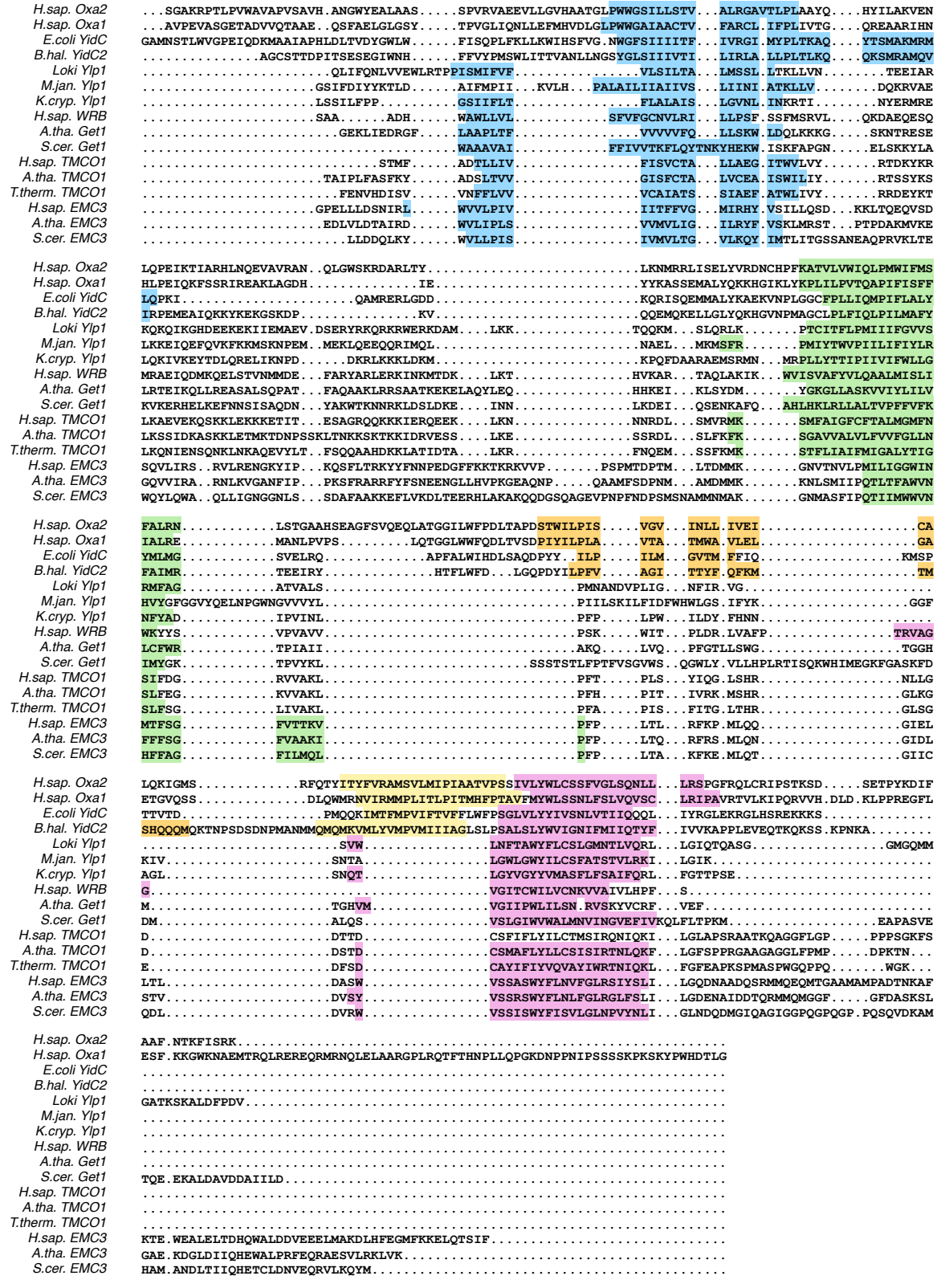


**Figure S1. Multiple sequence alignment of members of the Oxa1 superfamily, related to Figure 2.** PROMALS3D was used with standard parameters and without any user-defined constraints. TMD predictions from TOPCONS are highlighted; TMDs in the conserved core are colored as in Figure 2, and the additional two TMDs of the Oxa1/Alb3/YidC family are colored orange (TM3) and yellow (TM4).

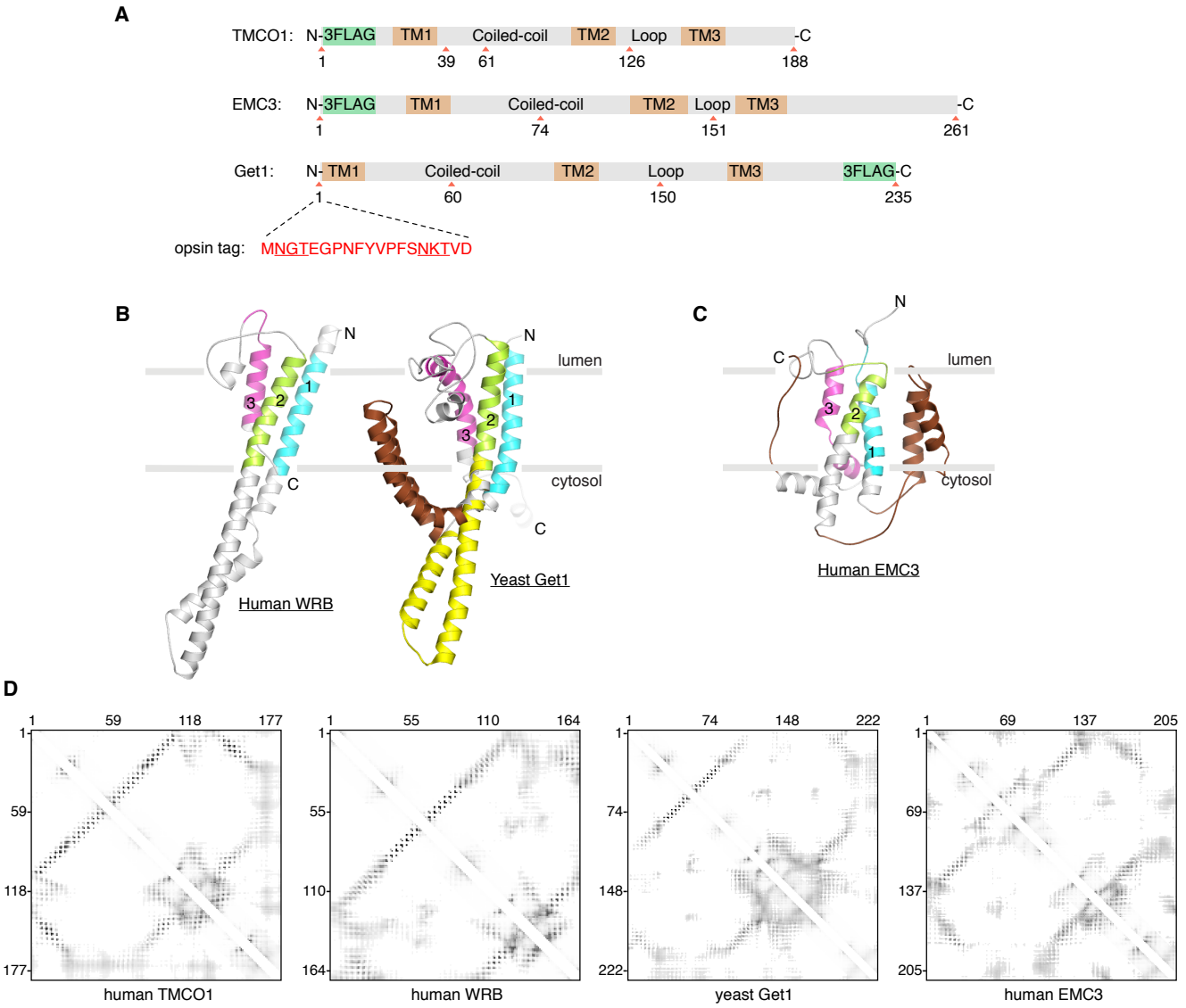


**Figure S2. Additional details for the topology mapping experiments and 3D modeling, related to Figure 2.** (**A**) Constructs used for glycosylation mapping. An opsin tag (red) containing two N-glycosylation sites (underlined) was inserted at the indicated positions of human TMCO1, human EMC3 and yeast Get1. Tag positions correspond to the native (untagged) sequence. For the TMCO1 and EMC3 constructs, a GSS linker connects the 3xFlag tag and the protein sequence. For the N-terminally opsin-tagged Get1 sequence, a 3xGSS linker was inserted before the first TMD, as sufficient distance from the membrane is required for effective glycosylation. (**B**) Co-variation-based 3D models of human WRB (left) and yeast Get1 (right), as in Figure 2D; note how the highly charged coiled-coil region of yeast Get1 (brown) bends back into the membrane bilayer (grey bars) in a non-physiologic conformation; this is likely due to the lack of a membrane bilayer energy term during 3D modeling (see Methods). In this case, a better, hybrid model is obtained by replacing the distorted coiled-coil (brown) with a crystallographically-defined Get1 coiled-coil (yellow; PDB 3ZS8) by manually docking it as a rigid body between TM1 and TM2 (see also Figure 2D). (**C**) Co-variation based 3D model of human EMC3 colored as in Figure 2D; a coiled-coil motif between TM1 and TM2, and the three TM core are both visible. However, similar to the yeast Get1 model, the coiled-coil and extended C-terminal region (both features colored brown) adopt physically implausible orientations in which they become embedded in the bilayer, despite being highly charged. (**D**) Heat maps of the RaptorX probabilities of two residues being in close proximity (<8 Å); higher probabilities are darker.

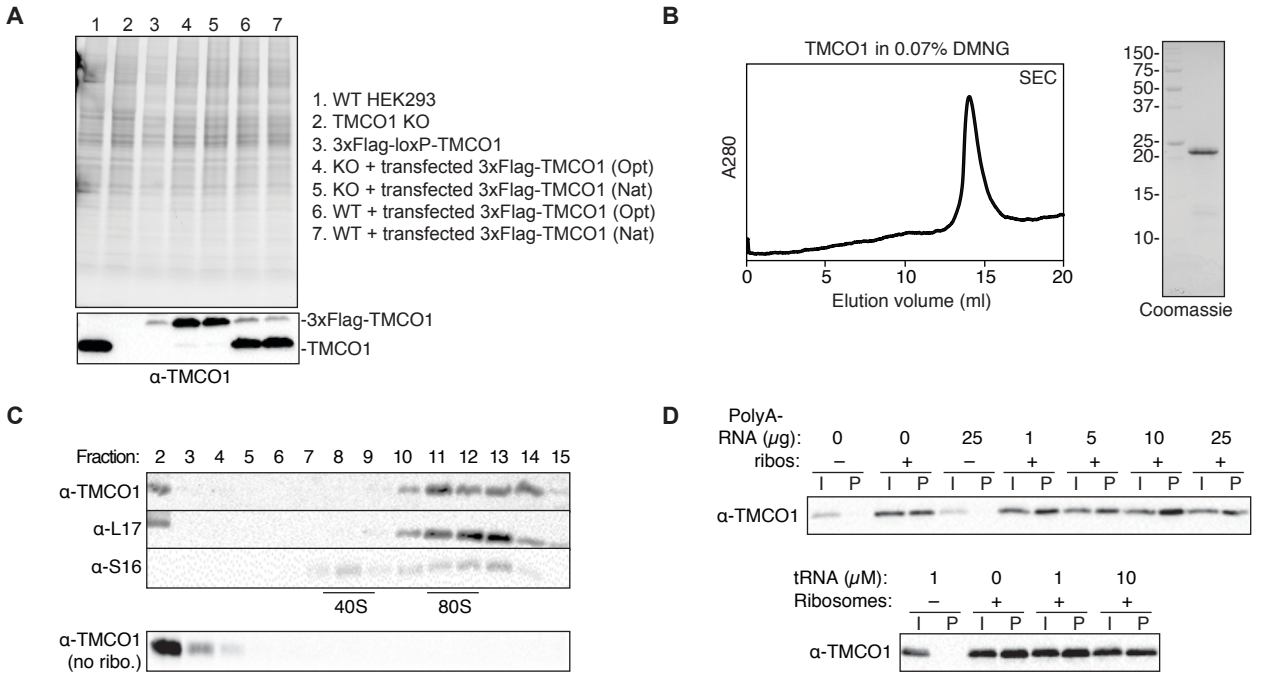


**Figure S3. Additional characterization of the ribosome binding properties of TMCO1 in cells and in vitro, related to Figure 3.** (**A**) Western blot analysis of TMCO1 expression levels in wild-type (WT) HEK293 cells, CRISPR/Cas9 generated knockout (KO) HEK293 cells, an integrated 3xFlag-tagged TMCO1 cell line and either KO or WT cells transfected with a 3xFlag-tagged TMCO1 construct either with ('Opt') or without ('Nat') codon optimization. A stain-free image of the gel prior to PVDF transfer shows that equal amounts of protein were loaded in each lane. Note that the transfected constructs express at lower levels than endogenous TMCO1 ('WT', lane 1). (**B**) Size-exclusion chromatography (SEC) of Ni-NTA affinity purified, recombinant TMCO1 in DMNG; pooled fractions are shown at right. (**C**) Sucrose gradient analysis of recombinant TMCO1 after chemical crosslinking to nuclease-treated rabbit reticulocyte lysate ribosomes. TMCO1 co-sediments with 80S ribosomes (but not the 40S ribosomal small subunit), while free TMCO1 remains at the top of the gradient. (**D**) Sedimentation analysis of TMCO1-ribosome complexes in the presence of excess competitor RNA; assays contained 1 μM TMCO1, 0.1 μM ribosomes and the indicated concentrations of competitor RNA.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Antibodies

Antiserum against human TMCO1 was generated by Lampire Biologicals. Rabbits were immunized with a KLH conjugated EKKKETITESAGRQQKK peptide, located in the cytosolic coiled-coil of TMCO1. Exsanguination bleed was supplemented with 0.02% sodium azide, flash-frozen in liquid nitrogen and stored at -80°C. For immunoprecipitation experiments, antibody was thawed and used immediately without further purification. For western blotting, initial experiments used unpurified serum; other experiments used peptide affinity purified antibody.

Antibodies against L17 (Abgent), S16 (Santa Cruz) and Derlin-1 (Abcam) were purchased, and antibodies against Sec61α and Sec61β were characterized previously (Gorlich et al., 1992).

### Cell culture

HEK293-Cas9 cells containing a 3xFlag-Cas9 construct integrated into the genome were generated from HEK293 Flp-In T-REx cells (Invitrogen). A *TMCO1* knockout line derived from these cells was generated at the Genome Engineering Core Facility at the University of Chicago, using a guide RNA with the sequence 5'-GAAACAATAACAGAGTCAGCTGG-3'. Cas9 expression was induced by addition of doxycycline at 10 ng/mL, followed by transfection of a gRNA-expressing plasmid. Single cells were then seeded into 96 well plates allowed to grow clonally. The final *TMCO1* knockout line was verified by both genomic DNA sequencing and immunoblotting with an α-TMCO1 antibody (Figure S3A).

A separate cell line containing an N-terminally Flag tagged TMCO1 was also generated at the same facility using a previously described two step strategy (Xi et al., 2015). The resulting cell line has one nonfunctional TMCO1 allele and one allele containing a 3xFlag-tagged TMCO1 with a 13 amino acid linker (ITSYNVCYTKLSG, from the Cre-lox recombination) before the TMCO1 ORF. The 3xFlag-TMCO1 lines were verified by both genomic DNA sequencing and immunoblotting with α-TMCO1 and α-Flag antibodies (Figure S3A).

Cells were grown in DMEM supplemented with 10% Fetal Bovine Serum (Gemini Benchmark; Lot #A99D00E) and penicillin/streptomycin mixture (Invitrogen). The culture medium was also supplemented with 15 µg/mL Blasticidin and 100 µg/mL Hygromycin B for the *TMCO1* knockout and 3xFlag-TMCO1 cell line generation procedure, but not when growing cells for other applications.

### Isolation of total membrane fraction from HEK293 cells

Cells were harvested at a density of 70-100% while growing. Media was removed and cells were scraped into DPBS. Cells were collected by 5 min at 500 x g centrifugation at 4°C, and then lysed osmotically (Sabatini, 2014) by resuspending in a volume of HM Buffer (10 mM Hepes pH 7.5, 10 mM potassium chloride, 1 mM magnesium chloride) equal to 3.5x the volume of the cell pellet. Cells were allowed to swell on ice for 15 minutes, followed by 15 strokes of a douncer

with a tight-fitting pestle (Kontess). Sucrose was then added to 250 mM to balance osmolarity. Nuclei were then removed by pelleting 3 minutes at 700 x g, and the supernatant was centrifuged 10 minutes at 10,000 x g to collect the membrane fraction. Contrary to previous studies, in our hands this was sufficient to pellet most biological membranes of interest, including the endoplasmic reticulum, Golgi, plasma membrane and mitochondria. The membranes were then washed with assay buffer (150 mM potassium acetate, 50 mM Hepes pH 7.4, 5 mM magnesium acetate) and centrifuged again 10 minutes at 10,000 x g to remove any residual cytosolic proteins.

Membranes used for sucrose cushions, gradients, and pull-downs were further treated with micrococcal nuclease to digest polysomes as follows: reaction was supplemented with calcium acetate to 1 mM and 100 Units of micrococcal nuclease (NEB), incubated 10 minutes at 25°C, and then quenched by addition of EGTA to 2 mM. Membranes were then washed again with assay buffer to remove nuclease.

**Recombinant TMCO1 production**

The gene encoding human TMCO1 was amplified by PCR from total human testicular cDNA (Biosettia), subcloned into a pET28b vector (Novagen) encoding an N-terminal 6xHis tag followed by a TEV protease site, and verified by DNA sequencing. TMCO1 encoding vectors were transformed into E. coli BL21(DE3) and colonies from these transformations were used to inoculated terrific broth (TB, Fisher) starter cultures in baffled flasks containing 50 µg/mL kanamycin. 50 mL starter cultures were grown overnight at 37°C and 250 rpm. 1 L TB cultures containing 50 µg/mL kanamycin were inoculated with 3 mL of starter culture, grown at 37°C, and shaken at 250 rpm until they reached an $A_{260}$ of 0.6. Expression was induced by addition of 0.1 mM isopropyl-β-d-thiogalactoside (IPTG, Sigma) and growth was continued for 4 hrs at room temperature and 250 rpm. Cells were harvested by centrifugation and pellets frozen at -80°C.

Frozen cell pellets were resuspended in 35 mL ice cold lysis buffer (500 mM NaCl, 50 mM Hepes pH 7.5, 10 mM imidazole pH 7.5, 20 µM EDTA pH 8, 1 mM PMSF, 2 mM DTT, 5% glycerol (v/v)) supplemented with 10 µg/mL DNaseI and 0.5 mg/mL of lysozyme. Resuspended pellets were dounced five times on ice and lysed by passages twice through a high pressure microfluidizer. Lysate was clarified by centrifugation at 18,500 x g for 45 min at 4°C. To pellet bacterial membranes, the crude lysate supernatant was subjected to centrifugation at 120,000 x g for 1 hr at 4°C. Pelleted membranes were resuspended gently with a paintbrush in 40 mL ice cold lysis buffer, supplemented with 1% Decyl Maltose Neopentyl Glycol (DMNG, Anatrace), and incubated overnight (~14 hrs) at 4°C with gentle end-over-end mixing. Detergent soluble material was isolated by centrifugation at 120,000 x g for 1 hr at 4°C and batch purified by TALON affinity chromatography (Clonetech). The column was washed with 10 column volumes of lysis buffer supplemented with 15 mM Imidazole pH 7.5 (25 mM Imidazole total) and 0.07% DMNG. Protein was eluted in elution buffer (500 mM NaCl, 50 mM Hepes pH 7.5, 2 mM DTT, 300 mM imidazole pH 7.5, 0.07% DMNG) and further purified by size exclusion

chromatography (Superdex 200, 10/300 GL, GE Healthcare) in 500 mM NaCl, 50 mM Hepes pH 7.4, 2 mM DTT, 0.07% DMNG at room temperature. Desired fractions were pooled and concentrated in a 50 kDa MWCO Amicon ultra centrifugal filter (Millipore). 10% glycerol was added before flash freezing and storage in aliquots at -80°C. Protein concentration was determined by Bradford assay.

**Assays for *in vitro* association of TMCO1 with ribosomes**

High-salt stripped ribosomes were prepared from rabbit reticulocyte lysate (Green Hectares Farm). After supplementing with 350 mM KCl, the lysate was layered on top of a high density, high salt sucrose cushion (1 M sucrose, 500 mM KCl, 50 mM Tris pH 7.4, 5 mM $MgCl_2$), and subjected to centrifugation at 250,000 x g for 2 hrs at 4°C (TLA100.3, Beckman-Coulter). After incubating the pellet with ribosome buffer (250 mM sucrose, 150 mM KCl, 50 mM Tris pH 7.4, 5 mM $MgCl_2$) for 1 hr on ice, KCl was added to 500 mM and ribosomes were again pelleted through a high density, high salt sucrose cushion. Ribosome pellets were gently resuspended in ribosome buffer, aliquoted, and flash frozen for storage at -80°C.

Ribosome binding assays were carried out in binding buffer (150 mM KCl, 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 0.07% DMNG), with 100 nM purified rabbit reticulocyte ribosomes and a 10-fold molar excess (1 μM) of purified, recombinant TMCO1 in a total volume of 100 μL. After incubating for 1 hr at 4 °C, 80 μL of the binding reaction was pelleted through a sucrose cushion (1 M sucrose, 150 mM KCl, 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 0.07% DMNG) for 2 hr 250,000 x g at 4°C (TLA100.3, Beckman-Coulter). Pellets were washed with 1 mL of ice cold water and resuspended in 40 μL of 1x lithium dodecyl sulphate sample buffer supplemented with 100 mM β-mercaptoethanol. Competition assays were performed as described above, but with the addition of either tRNA or polyA RNA at the indicated concentrations before incubation.

*In vitro* crosslinking was performed by adding fresh DSP (in DMSO) to a final concentration of 250 μM, followed by incubation for 10 minutes at room temperature. Reactions were quenched by the addition of Tris pH 7.4 to a final concentration of 100 mM, followed by an additional 10 min incubation on ice. NaCl was added to 500 mM to dissociate uncrosslinked TMCO1 from the ribosome. To separate ribosomal subunits after crosslinking, samples were incubated with 2 mM puromycin and 1 mM PMSF for 30 min on ice, then 20 minutes at 37°C. Crosslinked, puromycin-treated samples were separated by centrifugation through a high salt sucrose gradient (10-50% sucrose, 500 mM NaCl, 50 mM Hepes pH 7.5, 0.07% DMNG, 5 mM $MgCl_2$) at 130,000 x g (SW28.1, Beckman-Coulter) for 14 hrs at 4°C. 1 mL fractions were collected manually from the top of the gradient, TCA precipitated, and analyzed by SDS-PAGE.

## SUPPLEMENTAL REFERENCES

Gorlich, D., Prehn, S., Hartmann, E., Kalies, K.U., and Rapoport, T.A. (1992). A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. Cell *71*, 489-503.

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Xi, L., Schmidt, J.C., Zaug, A.J., Ascarrunz, D.R., and Cech, T.R. (2015). A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression. Genome Biol *16*, 231.