Heterogeneous translational landscape of the endoplasmic reticulum revealed by ribosome proximity labeling and transcriptome analysis

The endoplasmic reticulum (ER) is a nexus for mRNA localization and translation, and recent studies have demonstrated that ER-bound ribosomes also play a transcriptome-wide role in regulating proteome composition. The Sec61 translocon (SEC61) serves as the receptor for ribosomes that translate secretory/integral membrane protein-encoding mRNAs, but whether SEC61 also serves as a translation site for cytosolic protein-encoding mRNAs remains unknown. Here, using a BioID proximity-labeling approach in HEK293T Flp-In cell lines, we examined interactions between ER-resident proteins and ribosomes in vivo. Using in vitro analyses, we further focused on bona fide ribosome interactors (i.e. SEC61) and ER proteins (ribophorin I, leucine-rich-repeat–containing 59 (LRRC59), and SEC62) previously implicated in associating with ribosomes. We observed labeling of ER-bound ribosomes with the SEC61β and LRRC59 BioID reporters, comparatively modest labeling with the ribophorin I reporter, and no labeling with the SEC62 reporter. A biotin pulse-chase/subcellular fractionation approach to examine ribosome exchange at the SEC61β and LRRC59 sites revealed that, at steady state, ribosomes at these sites comprise both rapid- and slow-exchanging pools. Global translational initiation arrest elicited by the inhibitor harringtonine accelerated SEC61β reporter-labeled ribosome exchange. RNA-Seq analyses of the mRNAs associated with SEC61β- and LRRC59-labeled ribosomes revealed both site-enriched and shared mRNAs and further established that the ER has a transcriptome-wide role in regulating proteome composition. These results provide evidence that ribosomes interact with the ER membrane via multiple modes and suggest regulatory mechanisms that control global proteome composition via ER membrane-bound ribosomes.

The endoplasmic reticulum (ER) is a heterogeneous organelle composed of rough and smooth membrane domains, distinguished by the presence or absence of bound ribosomes, respectively, and a diversity of primary cellular functions including secretory/membrane protein biogenesis, lipid biosynthesis, and calcium storage (1–4). In addition to these defining features, the ER engages in communication with different organelles, including mitochondria, endosomes, and the plasma membrane (1, 5, 6). The sites of organelle–ER communication are marked by multiprotein assemblies that define areas of regional specialization (e.g. mitochondrial-associated membranes) and provide evidence for the spatial organization of the ER membrane proteome as a biochemical/biophysical mechanism to accommodate the various structural and functional properties of this critical organelle (7–10).

In addition to a dedicated function in secretory/membrane protein biogenesis, recent studies examining mRNA partitioning between the cytosol and ER compartments have revealed a mRNA transcriptome-wide role for the ER in translation (11–18). Although a function for the ER in the translation of cytosolic protein-encoding mRNAs has been considered for many decades (11, 19), biochemical and structural biology studies identifying the Sec61 translocon as both a ribosome receptor and protein translocation channel suggest that such a function would require alternative modes of ribosome association with the ER (20–25). Presumably, ribosomes bound to the ER by such alternative mechanisms engage mRNAs ER-localized via mechanisms other than the SRP pathway (14, 15, 18, 26, 27). In support of this view, in vitro studies have identified ribosome-binding activity for a number of ER-resident membrane proteins, including the oligosaccharyltransferase (OST) subunit ribophorin I, LRRC59 (p34), p180 (RRBP1), and recently, the ER stress sensor Ire1p (28–32). Although these candidate ribosome receptors display high binding affinity to ribosomes in vitro, little is known regarding the potential diversity of ribosome–ER protein interactions in vivo and how a multiplicity of ribosome receptors might contribute to mRNA transcriptome localization and translation on the ER.

That ribosomes may associate with the ER via Sec61 translocon-independent interactions raises additional questions regarding ribosome exchange on the ER (33, 34). In current views, ribosome exchange is translation-dependent; cytosolic ribosomes initiate the translation of secretory/membrane

inhibitor complex; NP-40, Nonidet P-40; DOC, deoxycholate; RT, room temperature; FDR, false discovery rate; TPM, transcript(s) per million; RPK, read(s) per kilobase; PDB, Protein Data Bank.
protein-encoding mRNAs and then undergo SRP-dependent trafficking to the ER, with ribosome release back to the cytosol occurring upon termination (35–37). In the case of ER-bound ribosomes translating cytosolic mRNAs, additional mechanisms of ribosome localization and exchange on the ER need to be considered. For example, ribosomes may undergo cytosol–ER exchange independently of translation. Advancing the understanding of this phenomenon is necessary to elucidate how cells partition ribosomes between the cytosol and ER and how that process is regulated.

Here, we utilized BioID proximity labeling to investigate ribosome-proximal localization of four proposed ER ribosome receptors (ribophorin I, Sec61β, LRRCS59, and Sec62) and to monitor ER–cytosol exchange of BioID-tagged ribosomes. BioID utilizes a mutant bacterial biotin ligase (BirA*) that releases a soluble amine-reactive biotin intermediate (biotin-AMP) from the active site, covalently tagging near-neighbor proteins and providing in vivo spatial information (38). Of the four candidate ribosome receptors examined, two (Sec61β and LRRCS59) labeled ER ribosomes. Intriguingly, the ribosomal protein-labeling patterns of the two reporters were distinct, consistent with distinct ribosome interaction sites for the two proteins. RNA-Seq analysis of mRNAs from the different ribosome populations revealed both highly enriched and shared transcripts. Taken together, these data are consistent with a higher-order spatial organization of translation on the ER and suggest mechanisms whereby the ER could serve a broad role in proteome expression.

**Results**

**Experimental overview**

Recent reports identify a mRNA transcriptome-wide function for ER-associated ribosomes in proteome expression (15–18). Given the central role of the Sec61 translocon as both the protein-conducting channel and ribosome receptor for membrane and secretory proteins (25, 39), we postulated that ER membrane proteins other than the Sec61 translocon participate in ribosome–ER interactions (23, 25, 40). In the studies reported here, BioID constructs of a subset of candidate ribosome interactors were engineered, doxycycline-inducible HEK293T Flp-In™ cell lines were generated, and ribosome interactomes were studied by subcellular fractionation combined with MS and RNA-Seq analyses of biotin-tagged ribosomes. For this study, we chose four ER membrane proteins implicated in ribosome association. The Sec61 translocon is an established ribosome receptor, so a Sec61β−BioID chimera was developed to report on the Sec61 translocon (23, 41, 42). Ribophorin I, a component of the OST, is proximal to translocating nascent chains and a Sec61 translocon interactor and thus a high probability candidate for the ribosome interactome analysis (28, 29, 43). Sec62, although relatively unstudied in mammalian systems, is orthologous to yeast Sec62, which participates in post-translational translocation. In mammalian systems, Sec62 has been demonstrated to function in ribosome binding, with binding interactions mapped to regions adjacent to the ribosome exit tunnel, and so was also selected for the reported screen (44, 45). LRRCS59, also relatively unstudied, was identified as a mammalian ribosome-binding protein through biochemical reconstitution approaches and chemical cross-linking, where it was demonstrated to reside near large ribosomal subunits (30, 46). An in vivo function for LRRCS59 in ribosome binding has not been established, although anti-LRRCS59 IgG and Fab inhibit ribosome binding and protein translocation in vitro (46). The four BirA* chimeras thus include well-studied complexes (e.g. Sec61 translocon (Sec61β) and OST (ribophorin I)) and proteins implicated in ribosome association but lacking an established function in mammalian cells (i.e. LRRCS59 and Sec62).

**Ribosome interactor–BioID chimeras are ER-localized and predominately label ER membrane proteins**

To assess the subcellular localization and proximity labeling activity of the ribosome interactor–BioID chimera introduced above, reporter cell lines were induced with doxycycline for 16 h with 50 μM biotin supplementation, and expression patterns were determined by immunofluorescence microscopy using antiserum directed against BirA*. Biotin-labeling patterns were examined by staining with a streptavidin–AF647 conjugate (Fig. 1A). A cell line containing the cloning vector backbone served as a negative control (empty vector). As depicted in Fig. 1A, all reporter cell lines displayed perinuclear reticular staining with the BirA antisera, consistent with an ER localization for all BioID chimeras. Streptavidin staining of proximal biotin-labeled targets mirrored the BirA*-staining patterns, suggesting that the primary interactomes are largely confined to the ER (Fig. 1, A and B). These findings were further validated in comparisons of the staining patterns for streptavidin and the resident ER membrane protein TRAPα (Fig. 1B). As with the data depicted in Fig. 1A, we observed extensive overlap of the streptavidin-staining pattern with that of TRAPα and little discernible tagging of cytosolic proteins.

The immunofluorescence data depicting reporter expression and biotin labeling (Fig. 1) were further evaluated in cell fractionation studies (Fig. 2). Using a previously validated sequential detergent fractionation protocol (47, 48), BirA* chimera (Fig. 2A) subcellular distributions were assessed by SDS-PAGE/immunoblot analysis. These data are depicted in Fig. 2B and demonstrate that all ER membrane protein reporters were recovered in the membrane fraction (M) and displayed SDS-PAGE mobilities consistent with their predicted molecular weights (Fig. 2A). Consistent with the immunofluorescence data shown in Fig. 1, overnight biotin labeling was highly enriched in the membrane fractions (M) (Fig. 2 (C and D), TRAPα as ER marker), with only modest labeling of cytosolic proteins (C) (Fig. 2 (C and D), β-tubulin as cytosolic marker). Interestingly, the biotin-labeling patterns of the membrane fractions (Fig. 2C) were distinct from one another, indicating that the BioID reporters reside in different protein environments. The relative paucity of biotinylated cytosolic proteins was unexpected, given that the reactive biotin-AMP intermediate diffuses from the BirA* active site to modify solution-accessible lysines (49).

Ribophorin I and Sec61β are subunits of oligomeric protein complexes. To determine if their BirA* chimeras assemble into
native oligomeric complexes, we compared the hydrodynamic behavior of the chimera with the respective natively expressed proteins by glycerol gradient velocity sedimentation (Fig. 3). As depicted, the sedimentation patterns of all chimeras and their respective native proteins were similar, suggesting that the chimera were appropriately assembled into native complexes (29). Comparison of the native protein expression in both BioID cell lines and the empty vector cell line are similar, suggesting that induced expression of the chimera does not disrupt membrane protein expression. In yeast, such chimeras complement genomic deletions of the parent gene, also indicative of native-like function (16). To further examine the incorporation of the chimeras into their respective complexes, we performed a streptavidin pulldown of biotinylated proteins from the membrane fraction of each cell line and blotted with antisera against the proteins indicated in Fig. 3B. As shown, the chimeras labeled known interactors within their respective complexes, and labeling was limited to the direct complex interactors. For example, the Sec61β chimera labeled Sec61α, whereas the ribophorin I chimera labeled the OST subunits STT3A/B. Additionally, we observed that the LRRC59 chimera labeled SRP54, a key component of the SRP, thus suggesting an in vivo role for LRRC59 in ER-associated translation. Combining this observation with that in Fig. 2B, that labeling is restricted to the membrane fraction, we conclude that the BirA* labeling radius is highly restricted, and thus if any of these proteins are in proximity or bound to ribosomes in vivo, this method might reveal that spatial relationship.

Proximity labeling of ER-bound ribosomes by candidate ribosome interactors

To examine proximity interactions between ribosomes and the BioID ribosome interactor reporters, we conducted biotin-labeling experiments, focusing our analyses on the ribosome fraction, which we enriched for by ultracentrifugation of the subcellular fractions. As ribosome exchange on the ER is thought to be functionally coupled to the translation cycle (35, 51), we sought to determine whether ribosome interactions might vary after many rounds of translation. To achieve this, we performed a biotin-labeling time course, isolated cytosol and membrane fractions, enriched for ribosomes, and analyzed ribosomal protein biotin decoration by streptavidin blotting (Fig. 4).

Ribosomal proteins, being highly basic and lysine-rich, should be highly receptive to BirA* labeling. However, SDS-PAGE analyses of the ribosome fractions revealed that of the two BirA* chimeras, Sec61β and LRRC59, that efficiently labeled ribosomes, only a subset of ribosomal proteins were targets for labeling (Fig. 4). As shown, the distinct ribosome-labeling patterns seen for the LRRC59 and Sec61β BioID reporters were evident within 0.5 h of the biotin addition and enriched in the ER-bound ribosome fraction over the labeling time course.
monitored. The labeling pattern and relative ratio of ER-to-cytosolic ribosome labeling, most evident in the LRRC59 BioID reporter cell line, did not vary substantially over the 6-h labeling time course of the experiments (Fig. 4). Ribosome labeling was observed at 6 h for the ribophorin I BioID chimera, although relatively modestly. Under these experimental conditions, ribosome labeling was not observed for the Sec62 BirA* chimera. In the case of the ribophorin I reporter, the ribosome–OST interaction may be too short-lived for efficient labeling. Consistent with this interpretation, recent cryo-EM studies of ER microsomes have reported two distinct Sec61 translocon environments distinguished by the presence or absence of OST, where it is noted that OST recruitment to the translocon may be transient, being present for the brief interval of N-linked sugar addition (23, 43). In addition, we cannot rule out the possibility that the ribophorin I and Sec62 chimera may be compromised in their ability to associate with the Sec61 translocon and thus to report on translocon-bound ribosomes (52).

Interestingly, ribosomal protein-labeling patterns evident at early time points intensified as a function of labeling time but did not diversify, suggesting a preferred spatial orientation of the BioID reporter–ribosome interface. This phenomenon is further characterized in the analysis depicted in Fig. 4B, which illustrates the time course of ribosome labeling for the BioID reporters. Notably, the kinetics of ribosomal protein labeling by the LRRC59 and Sec61β BioID reporters are similar, suggesting that the two reporters undergo similar interactions with membrane-bound ribosomes.

To determine whether the LRRC59 and Sec61β BioID reporters were labeling translationally active ribosomes and to assess whether biotinylation perturbed ribosome function, sucrose gradient sedimentation analyses were performed (Fig. 5). Subunit identification was confirmed by RNA gel analysis of 18S and 28S rRNA distributions. In the 40S subunit fractions, we identified one strongly labeled band (S-R1) for the LRRC59 reporter and two strongly labeled bands (S-B1/2) for the Sec61β reporter (Fig. 5A) and the inverse for the 60S subunit: two bands (L-R1/2) in the LRRC59 set and one band (L-B1) in the Sec61β analysis (Fig. 5A). We cannot comment on whether these bands represent more than one protein, but as they are reproducible patterns, we labeled each band as indicated to facilitate comparisons between experiments. In comparing the patterns of ammonium chloride–washed, puromycin-treated 80S ribosomes from the respective cell lines, we observed that the S-B1 and S-B2 bands are reduced upon ammonium chloride/puromycin treatment, leading us to believe these may be 40S-associated ER membrane proteins. As the biotin-tagged ribosomal bands were evident throughout the polysome profile shown in...
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**Fig. 3.** BioID reporter constructs display hydrodynamic behavior similar to native complexes in density gradient sedimentation analyses. **A**, detergent extracts of reporter and control (empty vector) cell lines were separated by glycerol density gradient ultracentrifugation. Immunoblots of gradient fractions were performed to determine the sedimentation patterns of the native complexes and the BioID chimera constructs. Reporter cell line identities are indicated to the left, and antibodies used are indicated to the right of each blot. The bottom panel depicts the native protein sedimentation patterns in an empty vector control cell line. Lane 5, centrifugation-clarified cell extract supernatant fraction; LF, load fraction representing the top 0.8 ml of the gradient. The numbered lanes refer to the remaining gradient fractions. The data are representative of two independent biological replicates.

**B**, biotinylated proteins from the membrane fraction from each cell line were isolated by streptavidin magnetic bead affinity capture and eluted by the addition of a biotin/SDS elution buffer. Immunoblot analyses of oligomeric subunits of the different ER membrane protein complexes were performed as an orthogonal validation of BioID chimera incorporation into known oligomers. Antibodies used are indicated to the right of the blots. The vertical black line is to indicate that the data are from separate SDS-polyacrylamide gels. Input percentages were based on extract volumes. Data are representative of two independent biological replicates.

Fig. 5, depicts plots of ribosome labeling kinetics and the ribosome exchange patterns in an empty vector control cell line. The data are representative of two independent biological replicates. Consistent with the overall streptavidin-labeling patterns, both BioID reporters labeled a common set of ribosomal proteins whose locations on the ribosome, seen in magenta in Fig. 5C, are regionally clustered. Intriguingly, the shared proteins distribute in regions adjacent to the peptidyl transferase and nascent peptide exit site (53). Two ribosomal proteins were highly enriched in only one data set. RPL17 (red), enriched in the Sec61β data set, is located near the nascent chain exit site and has been demonstrated to serve important roles in transmembrane domain sensing and signaling to the peptidyl transferase, a function consistent with its appearance in the Sec61β interactome (54, 55). RPS3A (orange), enriched in the LLRC59 data set, is located near the mRNA exit site and has been shown to interact with the transcription factor CHOP (56). These data are consistent with cryo-EM data depicting a spatially constrained interaction between the ribosome and the translocon, mirroring data shown for the Sec61β BioID reporter (25). The LLRC59 BioID reporter, a known poly(A) mRNA-binding protein, also resides in proximity to bound ribosomes, consistent with a potential function in coupling translating ribosomes to translocons (40, 57).

**Translation-dependent and -independent modes of ribosome exchange on the ER**

Extending from the data depicted in Fig. 4A, we examined ribosome exchange between the ER and cytosol compartments and the contribution of translation to such exchange. To this end, we established a pulse-chase assay where reporter cell lines were labeled with biotin for 1 h (pulse) followed by incubation in nonbiotin supplemented media for the indicated amount of time (chase). Fig. 6A depicts plots of ribosome labeling kinetics in control (continuous labeling, dashed line) and experimental (biotin removal at 1 h, solid line) conditions. The cessation of labeling upon biotin washout indicates that this approach is suitable for pulse-chase analyses. In the data reported in Fig. 6B, ribosome exchange for both reporters is composed of two pools, one exhibiting exchange in the first 30 min of chase and the second that remains membrane-associated after 1 h of chase. Subsequently, we interrogated ribosome exchange under different translational states, utilizing cycloheximide (Fig. 6C) and harringtonine treatment (Fig. 6D) to stall translating ribosomes and inhibit initiation, respectively (58, 59). Inhibitors were added at 1 h, when the biotin-supplemented medium was removed from the cells. Fig. 6C depicts the ribosome exchange by densitometric analysis of the streptavidin blots from cyclo-
heximide-treated cells for both cell lines. Here, we observed that the exchange behaviors of the labeled ribosomes were similar to the untreated control (Fig. 6B), with a fraction of the labeled ribosomes recovered in the cytosol and a fraction remaining membrane-bound. Notably, exchange patterns were also mirrored in the total ribosomal pools (Fig. S1). In contrast, upon the harringtonine block of translation initiation, we observed a divergence in behavior of the labeled ribosomes (Fig. 6D). Ribosomes labeled by the LRRC59 reporter were recovered in the cytosol to a lesser degree than those labeled by the Sec61β reporter. In the Sec61β reporter cells, ~15% of L-B1 remains on the membrane after 30 min of harringtonine treatment versus 35% of the large subunit protein band L-R2 in the LRRC59 reporter cells. Throughout treatment for each of these inhibitors, the two Sec61β bands S-B1 and S-B2 remain exclusively on the membrane. In summary, LRRC59-labeled ribosomes associate with the ER membrane in a manner that is relatively less dependent on translation (i.e., cycloheximide-insensitive exchange), whereas Sec61β-labeled ribosomes are sensitive to a global block in initiation, consistent with an SRP pathway–targeting model.

**Figure 4. Candidate ribosome-interacting BioID chimeras label distinct subsets of ribosomal proteins.** A, time course of ribosome labeling and biotinylated ribosome subcellular distributions in the BioID reporter cell lines. Shown are streptavidin blots and paired total protein analyses of ribosomes isolated by ultracentrifugation of cytosol (C) and membrane (M) fractions prepared as described in the legend to Fig. 2. For all reporter cell lines, reporter induction was performed for 16 h. Cytosol and membrane fractions were isolated at the indicated times after the biotin addition. Ribosome fractions were separated by SDS-PAGE, transferred to nitrocellulose membranes, and ribosomal protein patterns were determined by India ink staining followed by biotinylated protein detection by streptavidin blotting. B, quantification of the ribosomal signal intensity of the 25 kDa bands in the cytosol and membrane fractions in each blot, plotted against time after the biotin addition. Bars, signal range in two independent biological replicates. Ribosome-labeling patterns and subcellular distributions (A) are representative of >10 independent experiments.
Domain-specific RNA-Seq reveals regional mRNA enrichments

A primary objective of this study was to examine the translational landscape of the ER, including whether mRNAs and their translation are spatially organized, perhaps into clusters or translation centers. We have identified interactions between ER-bound ribosomes, the translocon subunit Sec61β/H9252, and the candidate ribosome receptor LRRC59. To extend these findings to mRNAs translated at these domains, biotin-tagged, ER-associated ribosomes were purified from the Sec61β and LRRC59 BioID cell lines, and the associated mRNAs were identified by RNA-Seq (Fig. 7). In brief, following a 4-h biotin-labeling period, the cytosolic, free ribosome fraction was released via digitonin extraction, the membrane-associated ribosomal fraction was solubilized in dodecylmaltoside, and the ribosomes were then separated from the co-solubilized membrane proteins by size-exclusion chromatography on Sephacryl S-400.
Biotinylated ribosomes, which are recovered in the S-400 void fractions, were isolated by avidin-magnetic bead capture, the total RNA fractions were isolated and depleted of rRNA, and cDNA libraries were prepared for deep sequencing. To correct for background mRNA contributions, parallel isolations were performed with the empty vector cell line, and cDNA libraries from these mock purifications were deep-sequenced in parallel. Three biological replicates are represented (Fig. S2).

As expected, coding RNAs comprised the majority of the counted reads (Fig. 7A). However, we observed an interesting
Figure 7. RNA-Seq analysis of Sec61β and LRRC59 BioID reporter-labeled ribosomes reveals divergent transcriptomes and demonstrates that ER-bound ribosomes engage in the translation of cytosolic and secretory protein-encoding RNAs. A, biotin-labeled ribosomes from the membrane fractions of Sec61β and LRRC59 BioID reporter cell lines were affinity-isolated, total RNA was extracted, and RNA-Seq analyses of mRNA-enriched RNA were performed. Depicted is the relative fraction of trimmed read counts for each of the data sets aligning to a human reference genome, binned to coding, noncoding, and 7SL RNA sequences. B, subcellular distributions of proteins encoded by mRNAs represented as a percentage of the total. Stack plots of RNA-Seq TPM reveal an enrichment for cell membrane and organelar protein-encoding mRNAs (DeepLoc1.0) compared with the total mRNA distribution by TPM for membrane and total cell (LocTree3), using data sets from Reid and Nicchitta (15) and the Human Protein Atlas, respectively. The category “Organelle” encompasses mRNAs whose encoded proteins are localized to the ER, Golgi, lysosomes/vacuoles, plasma membrane, and mitochondria. C, table of the top 10 genes by log2 -fold change value for genes either enriched in a given ribosome fraction or present in both (shared), color-coded to indicate -fold enrichment over the control data sets. D, bubble plots depicting the -log(FDR) of reporter cell line–enriched GO molecular function enrichments for the transcriptome identified by each chimera. Red line, an FDR cutoff of 0.05. Human GO term IDs are listed in Table S1. Analysis is shown from three biological replicates performed as independent experiments, with individual bar-coded libraries combined for multiplexed deep sequencing.
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difference in noncoding mRNAs between the data sets. For the purposes of this study, we focused on the noncoding 7SL RNA of the signal recognition particle (60). 7SL RNA is enriched over control in the BioID reporters, with a higher representation in the LRRC59 versus Sec61β data sets. Consistent with the 7SL RNA enrichment in the LRRC59 data set, SRP54, the 54-kDa SRP protein subunit, was enriched in the LRRC59 Western blotting in Fig. 3B and serves as orthogonal validation for the specific enrichment of SRP.

Fig. 7B depicts the relative transcript per million (TPM) distributions, sorted by the subcellular localization fate of the encoded protein, for the Sec61β- and LRRC59-enriched pools, as compared with the total mRNA transcriptome and total ER-associated mRNA transcriptome. As expected, the ER-associated mRNA transcriptome (Fig. 7B, Mem) differs substantially from the total cellular mRNA transcriptome by its enrichment in secretory- and membrane-protein-encoding transcripts, although with a substantial representation of cytosolic protein-encoding mRNAs (15–17). Intriguingly, the data sets from the BioID reporters are further enriched for mRNAs encoding organelle-associated proteins, which comprise over 60% of their data sets. This finding suggests that the BioID reporters reside in specific ER environments enriched in organelle-encoding versus secretory cargo-encoding mRNAs (Fig. 7C).

Displayed in Fig. 7D are the highest-confidence divergent GO biological process enrichments for the two reporter gene sets, which demonstrate that the two examined reporter domains display transcriptome specialization. For example, genes identified in the LRRC59 data sets are enriched in GO terms associated with cell contact and adhesion (GO: 00078742), wound healing (GO: 0009611 and 0042060), and blood coagulation (GO: 0007596). Conversely, Sec61β shows an enrichment for regulatory processes associated with signaling pathways (GO: 008285, 1901701, 0031348, 0071230, 0071407, and 0019218), reflecting the cytosolic and nuclear mRNAs associated with its data set. This finding corroborates previous findings from our laboratory that cytosolic mRNAs noncanonically associated with the ER are enriched in these GO categories (15). Intriguingly, for both translation domains, one of the most enriched genes is the parent reporter gene. Thus, ribosomes engaged in the translation of the reporter reside in proximity to their translation product (Fig. 7C). Such an intimate association may arise if the reporter parent genes encode or associate with an interacting RNA-binding protein. Consistent with this view, both LRRC59 and Sec61β have been identified as poly(A) RNA-binding proteins (61).

In summary, RNA-Seq analyses of mRNAs undergoing translation on ribosomes proximal to the Sec61β and LRRC59 BioID reporters revealed translational specialization, where specific GO category gene sets were enriched in the two domains, and shared translation functions, where numerous genes were common to the two translation domains. Combined with the ribosome exchange and ribosomal proteomic data reported above, these data suggest a higher-order organization and functional specialization of the ER.

Discussion

Here we report on the translational landscape of the ER from the biochemical perspective of candidate ribosome-interacting proteins. The rationale for this study is rooted in the growing number of reports demonstrating that cytosolic protein-encoding mRNAs undergo translation on ER-bound ribosomes. Indeed, recent analyses indicate that cytosolic protein-encoding mRNAs comprise the majority of the translation activity of total ER-bound ribosomes (15, 17, 62). These reports raise a number of intriguing questions regarding mechanisms and specificity for RNA localization to the ER as well as mechanisms of ribosome localization and exchange on the ER, which is accepted to be functionally linked to secretory/membrane protein synthesis (33). Indeed, there is a fundamental cell biology element to these questions, where a dedicated role for the ER in the biogenesis of secretory and membrane proteins is well-established, although the question of the exclusivity of this role has been posed for many decades and continues to be debated (11, 13, 15, 62). Here, we used an unbiased proximity labeling approach, BioID, to investigate the near-neighbor environments of both established and candidate ribosome-interacting ER membrane proteins, including Sec61β, a subunit of the Sec61 translocon; ribophorin I, which is a subunit of the OST complex and resides in proximity to Sec61 translocons (29); and LRRC59, which displays ribosome binding activity in vitro (30). We report four primary findings from these studies: (i) the ribosome interactors examined reside in interactome-distinguishable ER membrane protein environments; (ii) LRRC59 resides proximal to ER-bound ribosomes and thus likely contributes to the totality of ribosome association on the ER; (iii) ribosomes residing next to different reporters are engaged in the translation of both topogenic signal-encoding mRNAs and soluble protein-encoding mRNAs, including commonly shared and selectively enriched transcripts; and (iv) ribosome exchange rates are slow, relative to translation, and can be distinguished by exchange rates under different conditions of translational inhibition. Combined, these data suggest that multiple ribosome-interacting proteins contribute to ribosome association on the ER and, by virtue of their slow exchange rates, are spatially disposed to perform a general function in translation.

A largely unexpected observation from this work is that the biotin-labeled environments of the BioID reporters were heavily biased to ER membrane. We had predicted that membrane and cytosolic proteins would be similarly accessible to biotin modification. The bias to biotin conjugation of ER membrane proteins suggests that the labeling radius for the reactive biotin-AMP intermediate is highly restricted (63). Whereas the exact reason for this bias remains to be determined, we speculate that it reflects both high local concentrations of reactive sites and high residence lifetimes of ER membrane proteins proximal to the reporters. These observations are under further study, to more comprehensively examine evidence for higher-order organization of the ER membrane.

A particularly intriguing observation from these studies is the finding that LRRC59 is near translating ribosomes. Although LRRC59 had previously been reported to function as a ribosome-binding protein in vitro, a function in ribosome
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association in vivo has not been demonstrated (30, 46, 64). Indeed, after a decades-long search for the ribosome receptor, which yielded the identification of the ribophorins LRRC59, p180, and Sec61α, among others, research interest has largely focused on the Sec61 complex as the sole ribosome-interacting ER protein (23, 25, 39, 65). Substantial structural data support this functional assignment, but these data do not exclude the possibility that additional ER proteins contribute to ribosome association with the ER (44, 66–69). In support of this view, many ER membrane proteins, including LRRC59, have been found to bind poly(A) RNA, including MTDH (AEG-1), which we recently demonstrated to be an ER RNA-binding protein enriched in membrane protein–encoding mRNAs (70), p180 and CKAP4, which have also been demonstrated to be mRNA-binding proteins (27, 57, 68). Taken together, these findings suggest that LRRC59 may have a role in coupling ER-associated translation to translocon engagement of the translation product. These data fit with a previously proposed model suggesting that ribosome-interacting proteins might diffuse in the ER membrane to allow nascent chain engagement with unoccupied translocons (40, 71, 72).

Because the two BioID reporter chimeras, Sec61β and LRRC59, tagged ER-bound ribosomes in readily distinguishable patterns, we were interested in determining whether such differences were indicative of a transcriptome organization to the ER. As noted, a primary role for the ER in secretory/membrane protein biogenesis is well-established by the many studies examining the subcellular distributions of mRNAs between the cytosol and ER compartments (15, 18). Although the interpretation of these data has been debated, studies of mRNA distributions between the cytosol and ER compartments in yeast, by ER-localized BirA-AVI tag labeling or by SRP-directed immunoprecipitation, demonstrate that many cytosolic protein-encoding mRNAs display log₂ cytosol enrichments of <1–2 and are thus substantially represented on the ER (16, 17). This mRNA distribution is similar to data reported in mammalian cells (15, 18). In the current study, we examined the associated transcriptomes of biotin-tagged, ER-bound ribosomes. As with earlier studies, we report that although enriched in secretory/membrane protein-encoding mRNAs, ribosomes residing in proximity to both the Sec61β and LRRC59 BioID chimera contained a fraction of cytosolic protein-encoding mRNAs. The RNA populations for the two cell lines displayed both shared and enriched transcripts, and, of high interest, the enriched transcript cohorts differed in GO enrichments, with the Sec61β cohort being enriched in mRNAs encoding ER proteins and the LRRC59 cohort being enriched in mRNAs encoding integral plasma membrane proteins. Particularly interesting was the finding that one of the most enriched transcripts for both reporters was the “self-mRNA.” These findings support the concept of translation centers on the ER, where mRNAs encoding proteins of related function are coordinately translated in a coherent, localized manner. It remains to be determined how individual mRNAs are targeted to distinct sites or whether mRNAs may be directly recruited to such sites via binding interactions with additional ER RNA-binding proteins, such as AEG-1, or by stably associated ribosomes potentially with heterogeneous composition (70, 73–76).

Whereas there is a significant overlap of transcripts between the two reporters, the two ribosome populations showed differences in exchange upon global shutdown of translation initiation where a subset of LRRC59-labeled ribosomes remain bound to the membrane, whereas ribosomes labeled by Sec61β are released entirely to the cytosol and unable to regain access to the membrane (Fig. 6D). These data raise two interesting points: (i) subsets of ribosomes appear to require different mechanisms of membrane association, and (ii) subsets of ribosomes are restricted to a specific membrane environment potentially through heterogeneity of the ribosome interactome within the cell. The latter can be inferred by the fact that membrane-bound ribosomes that leave to the cytosol do not return potentially due to the crucial function of initiation in the SRP pathway. Further identifying the mechanism of ribosome association via LRRC59 and its apparent role in membrane-bound translation awaits further study.

In summary, we present biochemical and transcriptomic data supporting the view that translation on the ER is subject to higher-order organization, where cohorts of ribosomes and their respective mRNAs are localized to distinct spatial environments. These data also provide additional evidence in support of a transcriptome-wide function for the ER in translation. The remaining questions are many, but given emerging data on the nanoscale structural organization and translational organization of different regions and compartments of the cell, notably dendrites, mitochondria, stress granules, and P bodies, these data are consistent with higher organization of transcriptome expression and regulation as an evolutionarily conserved cellular strategy (1, 9, 77–80).

Experimental procedures

Generation of BioID chimera

Plasmids were from the following sources: pCMV-Sport6-RPN1 (Transomic ID: pCS6-BC010839, TransOMIC, Huntsville, AL), pCMV-Sport6-LRRC59 (Transomic ID: pCS6-BC017168), Sec61β (Transomic ID: pOTB7-BC BC001734), Neo-IRES-GFP-Sec62 (Richard Zimmerman, Saarland University, Homburg, Germany), pEYFP-N1-BirA* (Scott Soderling, Department of Cell Biology, Duke University Medical Center). Gibson assembly master mix (New England Biolabs (Ipswich, MA), E2611S) was used with the specified amplified fragments to generate all constructs with the indicated BirA* and a Gly-Ser-Gly-Ser linker between the protein of interest and the BirA*. All resulting constructs were cloned into pcDNA5-FRT/TO for downstream generation of HEK293 Flp-In T-REx cell lines (Thermo Fisher Scientific). The BioID tags were placed on the terminus facing the cytosol; for Sec62, we chose the C terminus to avoid disrupting proposed ribosome interactions (44). All primer sequences are listed in Table S2. All chimera sequences were confirmed by direct sequencing using CMV-Forward and BGH-Reverse sequencing primers supplied by Eton Biosciences (Research Triangle Park, NC).

Generation of HEK293 Flp-In T-REx cell lines

HEK293 Flp-In T-REx cell lines were generated according to the manufacturer’s instructions (Thermo Fisher Scientific). Cells were transfected in 6-well culture dishes at 80% confluent
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Expression of BirA fusion proteins

Expression levels were examined by doxycycline (Sigma-Aldrich, D9891) titration, and the following doxycycline concentrations were used for each construct: 10 ng/ml LRRC59-BioID, 5 ng/ml Sec61β-BioID, 50 ng/ml ribophorin I-BioID, 100 ng/ml Sec62-BioID. Expression of BioID constructs by doxycycline was performed for at least 16 h before the addition of 50 μM biotin unless otherwise noted.

Immunofluorescence analyses

Cells were plated on polylysine-coated 22-mm #1.5 coverslips (Globe Scientific (Paramus, NJ), 1404-15). Reporter expression was induced by doxycycline addition with 50 μM biotin added for an overnight labeling. After 16 h, cells were washed twice with PBS, fixed in 4% paraformaldehyde for 10 min on ice and 10 min at room temperature, and then washed three times with PBS for 5 min each. Cells were permeabilized with a blocking solution of 3% BSA and 0.1% saponin (Sigma-Aldrich, S-2149) in PBS for 1 h at room temperature. Primary staining was performed in identical solution supplemented with 1:200 BirA antibody (Sino Biological Inc. (Wayne, PA), rabbit IgG) or 1:50 TRAPα antibody (81) (polyclonal, rabbit IgG) at 4 °C overnight. Following five 3-min washes of 0.1% saponin in PBS, coverslips were incubated with 1:200 goat anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher Scientific, A-11034), 1:1000 streptavidin–Alexa Fluor 647 (Thermo Fisher Scientific, S21374), and 1:10,000 4′,6-diamidino-2-phenylindole (0.5 mg/ml stock solution) mixed in blocking solution at room temperature for 45 min in the dark. Coverslips were washed five times as above, rinsed and mounted in FluorSave hard mount (Calbiochem, 345789), and cured at 4 °C overnight prior to imaging.

Fluorescence imaging

All imaging was performed on a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA) equipped with a ×100, numerical aperture 1.4 oil immersion objective (UPlanSApo 100XO, Olympus (Tokyo, Japan)) and a high-resolution CCD camera (CoolSNAP HQ2, Photometrics (Tucson, AZ)). Images were acquired as z-stacks (at 0.2-μm intervals) at identical exposure conditions across the samples for a given protein. The data were deconvolved using the C program (Applied Precision, Mississauga, Canada) and processed further on ImageJ-FIJI software to render maximum intensity projections (as required), merge channels, and pseudocolor the images. Only linear changes were done to the brightness/contrast values of the images, as required, and such changes were applied uniformly across all images in a given experiment.

Sequential detergent fractionation and general cell lysis

Cells were washed twice with ice-cold PBS containing 50 μg/ml cycloheximide (CHX) (VWR Scientific (Radnor, PA), 94271) for 3 min each wash. Permeabilization buffer (110 mM KOAc, 25 mM HEPES, pH 7.2, 2.5 mM Mg(OAc)2, 0.03% digitonin (Calbiochem, 3004010), 1 mM DTT, 50 μg/ml CHX, 40 units/ml RNAseOUT (Invitrogen, 10777-019), protease inhibitor complex (PIC) (Sigma-Aldrich, P8340) was added to cells, and incubations were performed for 5 min at 4 °C. The supernatant fraction (cytosol) was collected, and cells were rinsed with wash buffer (110 mM KOAc, 25 mM HEPES, pH 7.2, 2.5 mM Mg(OAc)2, 0.004% digitonin, 1 mM DTT, 50 μg/ml CHX, 40 units/ml RNAseOUT, PIC). Cells were then lysed in NP-40 lysis buffer (400 mM KOAc, 25 mM HEPES, pH 7.2, 15 mM Mg(OAc)2, 1% NP-40, 0.5% DOC, 1 mM DTT, 50 μg/ml CHX, 40 units/ml RNAseOUT, PIC, for 5 min at 4 °C. Both cytosolic and membrane fractions were cleared by centrifugation (15,300 × g for 10 min). Total cell lysis was performed in the ER lysis buffer by incubating cells at 4 °C for 10 min. Lysates were cleared by centrifugation as above.

Western blotting

Lysate protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, 23225). SDS-PAGE was performed in 12% acrylamide gels containing 0.5% trichloroethanol. Gels were UV-irradiated for 5 min and imaged using an Amersham Biosciences Imager 600 (GE Life Sciences) to verify protein loading. Gels were then equilibrated in Tris-glycine transfer buffer for 5 min and transferred using a Trans Blot SD semi-dry transfer apparatus (Bio-Rad). Blots were blocked in PBS, 3% BSA for 1 h before primary antibody was added at the indicated dilution and incubated for 2 h at RT or overnight at 4 °C. Goat secondary antibodies (LI-COR Biosciences, Lincoln, NE) were matched to the species of the primaries used and diluted 1:10,000. Streptavidin was used at a dilution of 1:20,000. Secondary reagents were incubated for 45 min, washed five times with TBST, and imaged on the Odyssey CLx (LI-COR Biosciences). Primary antibodies used were as follows: BirA (Abcam, catalog no. 14002, polyclonal, chicken IgG), TRAPα (81) (polyclonal, rabbit IgG), tubulin (Iowa Hybridoma Bank (Iowa City, IA), E7, monoclonal, mouse IgG), Sec61β (gift of Ramanujan Hegde, University of Cambridge, polyclonal, rabbit IgG), LRRC59 (Bethyl Laboratories (Montgomery, TX) A305-076A, polyclonal, rabbit IgG), Sec62 (gift from Richard Zimmerman, polyclonal, rabbit IgG), ribophorin I (81) (polyclonal, rabbit IgG), streptavidin-RD680 (LI-COR Biosciences, P/N 925-68079).

RNA extraction

As adapted from Chomczynski and Sacchi (82), RNA was extracted from 1 volume of lysate using 2 volumes of GT buffer to 0.5 volume of water-saturated phenol, pH 4.5, and incubated for 5 min at RT before adding 0.8 volume of chloroform. Following centrifugation for 15 min at 10,000 × g, 4 °C for 15 min, the aqueous phase was recovered, and RNA was precipitated by
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the addition of 1.2 volumes of isopropyl alcohol and 0.15 volume of 3 M sodium citrate, pH 5.2. Following incubation at -20 °C for 1 h, RNA was recovered by centrifugation at 10,000 × g at 4 °C for 20 min. RNA pellets were washed in 70% ethanol, dried, and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was examined by denaturing formaldehyde gel electrophoresis.

Glycerol gradients

As adapted from Nikonov et al. (50), reporter construct-expressing BioID lines were lysed in 1 ml/10-cm dish of homogenization buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 1.5% digitonin, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM DTT, PIC) for 30 min at 4 °C. Lysates were cleared by centrifugation in a TLA 100.2 rotor at 40,000 rpm for 10 min, 4 °C (TL-100 Ultracentrifuge, Beckman Coulter, Brea, CA). 850 μl of the supernatant was then loaded onto a 8–30% glycerol gradient and centrifuged in an SW-41 rotor at 35,000 rpm for 15.5 h at 4 °C (L5-50B ultracentrifuge, Beckman). Gradients were fractionated into 12 fractions using a Teledyne Isco gradient fractionation system, and 15 μl of each fraction was analyzed by immunoblot.

Polysome gradients

Cells expressing BioID constructs were lysed in 50 mM HEPES, pH 7.2, 200 mM KOAc, 1 mM DTT, 2% dodecylmalto- side (ChemIlmex International Inc., Wood Dale, IL), 5 mM EGTA, PIC, 1 mM DTT, 50 μg/ml CHX for 10 min at 4 °C. Cell lysates were cleared at 15,300 × g for 10 min at 4 °C. 0.8 ml of lysate was loaded onto 15–50% sucrose gradients and centrifuged for 3 h at 35,000 rpm at 4 °C (L5-50B ultracentrifuge, Beckman). Gradients were fractionated into 12 fractions using a Teledyne Isco (Lincoln, NE) gradient fractionation system and analyzed by immunoblotting and denaturing RNA gel electrophoresis.

Biotin pulldowns

This method was adapted from Firtat-Karalar and Stearns (83). Constructs were expressed as above, with biotinylation reactions performed for 3 h prior to sequential detergent fractionation. The membrane fraction was obtained and volume-adjusted to a protein concentration of ~1.3 mg/ml and diluted 1:1 with 100 mM NaCl, 50 mM HEPES, pH 7.4, to reduce detergent concentrations. Pierce NeutrAvidin agarose (Thermo Fisher Scientific, 29200) resin was blocked for 1 h with 1% BSA and washed three times in HEPES buffer. Pulldown reactions were performed overnight at 4 °C. Beads were washed with the following buffers twice each for 10 min at RT: Buffer 1, 2% SDS in 50 mM HEPES, pH 7.2; Buffer 2, 0.1% DOC, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES, pH 7.5; Buffer 3, 0.5% DOC, 0.5% NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris, pH 7.4. Beads were then suspended in 50 μl of biotin elution buffer, vortexed, and heated for 15 min at 70 °C. Supernatant fractions were combined and concentrated to 50 μl in a Savant SpeedVac concentrator (Thermo Fisher Scientific).

Ribosome pulldowns

Cells were washed with PBS and lysed in NP-40 lysis buffer (as above). Lysates were cleared at 15,300 × g for 10 min, and the supernatant fraction was overlaid onto a 1 M sucrose cushion (2:1, load/cushion). Samples were centrifuged at 80,000 rpm for 25 min (TLA 100 rotor in a TL-100 ultracentrifuge, Beckman). Ribosome pellets were washed with PBS before being suspended in 50 mM HEPES, pH 7.4, 100 mM NaCl, 1% SDS, 10 mM EDTA, 1 mM DTT, by Dounce homogenization. Ribosome concentration was determined by the A₅₉₀ absorbance and calculated using the extinction coefficient: 5 × 10⁷/cm/M (84). Equal amounts of ribosomes were used for pulldowns, as above. Binding reactions were performed by end-over-end mixing for 90 min at room temperature with added Dynabeads M-270 streptavidin beads (Thermo Fisher Scientific, 65305). Beads were washed as above and suspended in 20 μl of HEPES buffer and submitted to the Duke Proteomics Core for on-bead digestion.

Mass spectrometry

On-resin trypsin digestion—The Dynabead complexes in solution were washed three times with 500 μl of 50 mM ammonium bicarbonate (AmBic) (Millipore Sigma, Burlington, MA). 20 μl of 1.0% acid labile surfactant (RapiGest, Waters, Milford, MA) in AmBic was added to each sample followed by an additional 20 μl of AmBic. Samples were subsequently reduced with 10 mM DTT (Millipore Sigma) for 30 min at 40 °C with shaking and alkylated using 20 mM iodoacetamide (VWR Scientific) for 30 min at room temperature. Digestion was performed using 500 ng of sequencing grade trypsin in AmBic (5 μl at 0.1 μg/μl; Promega, Madison, WI), at 37 °C overnight with shaking. Peptides were extracted by decanting supernatant into a separate 1.5-ml Eppendorf (Hamburg, Germany) LoBind tube and washing the resin with 50 μl of additional AmBic, which was also combined with digested peptides. The combined extract was acidified to 1% (v/v) trifluoroacetic acid (Thermo Fisher Scientific), heated to 60 °C for 2 h to cleave the RapiGest surfactant, and lyophilized to dryness.

Qualitative analysis of on-resin digested samples—All on-resin samples were resuspended in 20 μl of 1:2:97 (v/v/v) TFA/MCN/water. The samples were analyzed by nano-LC-MS/MS using a Waters nanoAcquity LC interfaced to a Thermo Q Exactive Plus via a nanoelectrospray ionization source. 2 μl of each on-resin sample was injected for analysis. Each sample was first trapped on a Symmetry C18, 300 μm × 180-mm trapping column (5 μl/min at 99:9:0.1 (v/v) H₂O/MCN for 5 min), after which the analytical separation was performed using a 1.7-μm ACQUITY HSS T3 C18 75 μm × 250-mm column (Waters). The peptides were eluted using a 90-min gradient of 5–40% MeCN with 0.1% formic acid at a flow rate of 400 nI/min with a column temperature of 55 °C.

Data collection on the Q Exactive Plus mass spectrometer was performed with data-dependent acquisition MS/MS, using a 70,000 resolution precursor ion (MS1) scan followed by MS/MS (MS2) of the top 10 most abundant ions at 17,500 resolution. MS1 was performed using an automatic gain control target of 1e6 ions and maximum ion injection time of 60 ms.
MS2 used an automatic gain control target of 5e4 ions, 60-ms maximum ion injection time, 2.0 m/z isolation window, 27-V normalized collision energy, and 20-s dynamic exclusion. The total analysis cycle time for each sample injection was ~2 h.

**Database searching**—Proteome Discoverer (Thermo Fisher Scientific) was used to generate mgf files from the data-dependent acquisition analyses, and the data were searched using Mascot version 2.5 (Matrix Science) with a custom database containing the human proteome downloaded from Uniprot combined with common proteins found in BirA experiments and common contaminants. The data were searched using trypsin enzyme cleavage rules and a maximum of four missed cleavages; fixed modification carboxymethylated cysteine; and variable modifications biotinylated lysine, deamidated asparagine and glutamic acid, and oxidated methionine. The peptide mass tolerance was set to ±5 ppm, and the fragment mass tolerance was set to ±0.02 Da. False discovery rate control for peptide and protein identifications was performed using Scaffold version 4 (Proteome Software, Inc.).

Analysis of scaffold data—Spectral counts were retrieved for only ribosomal protein hits with 90% protein identity, 50% peptide identity with at least two peptides (protein FDR 0.05, 379 proteins). Only ribosomal proteins were used for further analysis. Each experiment data set was divided by the control, and those exceeding a 2-fold difference were further analyzed. For each candidate, sample spectral counts were divided by the control, and proteins with a greater than 2-fold difference were termed “enriched” and those below the cutoff were termed “shared.” Those proteins with the same term between the two data sets were kept and mapped onto PDB file 3J7R, of the translating ribosome on the translocon.

**Biotinylated polysome isolation and sequencing**

Ribosomes were purified from the membrane fractions of sequential detergent fractionation of the indicated BioID cell lines by gel filtration chromatography, collecting the void fraction of a Sephacryl S400 column operating at a flow rate of 0.7 ml/min. Dynabeads M-270 streptavidin beads (Thermo Fisher Scientific, 65305) and 0.05% Triton X-100 were added to each sample and incubated overnight at 4 °C. Beads were washed three times for 10 min at 4 °C in high-salt wash buffer followed by suspension in low-salt buffer and extraction of bound RNA using an RNEasy kit (Qiagen (Hilden, Germany), 74104). RNA was quantified by Bioanalyzer 2100 analysis (Agilent, Santa Clara, CA), and like samples were combined to provide 10 ng of total RNA. RNA samples were concentrated to 12 μl using the E.Z.N.A. MicroElute RNA Cleanup Kit (Omega Bio-Tek (Norcross, GA), R6247), and libraries were constructed using New England Biolabs Ultra II RNA library kits (New England Biolabs, E7645S).

**Illumina Hi-Seq**

Libraries were submitted to the Duke Sequencing and Genomic Technologies for sequencing. The concentration of each library was estimated by a Qubit assay and run on an Agilent Bioanalyzer for library size estimation. Libraries were then pooled into equimolar concentration. The final pool was clustered on a HiSeq 4000 flow cell. Sequencing was done at 50-bp single read. bcls files generated by the sequencer were then converted into fastq files using Illumina bcl2fastq version 2.20.0.422, and reads were demultiplexed using the molecular indexes incorporated during library preparation.

**Sequencing analysis**

FASTA files were adapter-trimmed using Trimmomatic version 0.32 (85), aligned to the human genome, build GRCh38/h38, using HISAT2 0.5 default options for unpaired reads (86). Aligned read files were then counted using htseq-count version 0.5.4p3 (87) using options for nonstranded reads, intersection-strict mode, and “exon” as the feature to be counted using a UCSC hg38 GTF annotation file. This GTF file with unique gene IDs and transcript IDs was generated to a genePred file for hg38 using the genePredToGTF script from kentUtils. Data sets from the two cell lines were analyzed for differential expression versus the control experiments using DESeq2 version 1.18.1 (88). Gene lists were generated by taking the subset with ≥2-fold change over the control data set with an adjusted p value of 0.05 (89). Genes coding for protein products were selected for interaction and GO analysis using the STRING database. Localization predication analysis was performed using the DeepLoc1.0 Profiles algorithm (90). TPM analysis was performed by first calculating reads per kilobase (RPK), summing the RPK values, and dividing by 1 million to use as the scaling factor. Individual RPK values were divided by the scaling factor to obtain a gene-specific TPM value for the given subset of data for better comparison of the data sets.

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**References**

1. English, A. R., and Voeltz, G. K. (2013) Endoplasmic reticulum structure and interconnections with other organelles. *Cold Spring Harb. Perspect. Biol.*, 5, a013227 CrossRef Medline
mRNA transcriptome localization on the endoplasmic reticulum

2. Schwarz, D. S., and Blower, M. D. (2016) The endoplasmic reticulum: structure, function and response to cellular signaling. Cell Mol. Life Sci. 73, 79–94 CrossRef Medline

3. Fawcett, D. W. (1966) An Atlas of Fine Structure: The Cell, Its Organelles, and Inclusions, Reprint Edition, pp. 303–330, WB Saunders Co., Philadelphia, PA

4. Lynes, E. M., and Simmen, T. (2011) Urban planning of the endoplasmic reticulum (ER): how diverse mechanisms segregate the many functions of the ER. Biochim. Biophys. Acta 1813, 1893–1905 CrossRef Medline

5. Valm, A. M., Cohen, S., Legant, W. R., Melnins, J., Hershberg, U., Wait, E., Cohen, A. R., Davidson, M. W., Betzig, E., and Lippincott-Schwartz, J. (2017) Applying systems-level spectral imaging and analysis to reveal the organelle interactome. Nature 546, 162–167 CrossRef Medline

6. Helle, S. C. I., Kanfer, G., Kolar, K., Lang, A., Michel, A. H., and Kornmann, B. (2013) Organization and function of membrane contact sites. Biochim. Biophys. Acta 1833, 2526–2541 CrossRef Medline

7. Ding, J., Le, S., Sun, A., Quintana, A., Ding, Y., Ma, G., Tan, P., Liang, X., Zheng, X., Chen, L., Shi, X., Zhang, S. L., Zhong, L., Huang, Y., Dong, M.-Q., et al. (2015) Proteomic mapping of ER–PM junctions identifies STIM1 as a regulator of Ca^{2+} influx. Nat. Cell Biol. 17, 1339–1347 CrossRef Medline

8. Hung, V., Lam, S. S., Udeshi, N. D., Svinkina, T., Guzman, G., Mootha, V. K., Carr, S. A., and Ting, A. Y. (2017) Proteomic mapping of cytosolic ribosomes and nascent polypeptides during translocation. Proc. Natl. Acad. Sci. U.S.A. 114, 3475–3482 CrossRef Medline

9. Vacek, J. E. (2014) MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. Biochim. Biophys. Acta 1841, 595–609 CrossRef Medline

10. de Brito, O. M., and Scorrano, L. (2010) An intimate liaison: spatial organization of the endoplasmic reticulum-mitochondria relationship. EMBO J. 29, 2715–2723 CrossRef Medline

11. Mueckler, M. M., and Pitot, H. C. (1981) Structure and function of rat liver mRNA transcriptome localization on the endoplasmic reticulum. J. Biol. Chem. 256, 1645–1650 CrossRef Medline

12. Diehn, M., Eisen, M. B., Botstein, D., and Brown, P. O. (2000) Large-scale identification of secreted and membrane-associated gene products using DNA microarrays. Nat. Genet. 25, 58–62 CrossRef Medline

13. Diehn, M., Bhattacharya, R., Botstein, D., and Brown, P. O. (2006) Genome-scale identification of membrane-associating human mRNAs. PLoS Genet. 2, e11 CrossRef Medline

14. Stephens, S. B., and Nicchitta, C. V. (2008) Divergent regulation of protein synthesis in the cytosol and endoplasmic reticulum compartments of mammalian cells. Mol. Biol. Cell 19, 623–632 CrossRef Medline

15. Reid, D. W., and Nicchitta, C. V. (2012) Primary role for endoplasmic reticulum-bound ribosomes in cellular translation identified by ribosome profiling. J. Biol. Chem. 287, 5518–5527 CrossRef Medline

16. Jan, C. H., Williams, C. C., and Weissman, J. S. (2014) Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. Science 346, 1257521 CrossRef Medline

17. Charton, J. W., Hunt, K. C. L., and Frydman, J. (2016) Cotranslational signal-independent SRP preloading during membrane targeting. Nature 536, 224–228 CrossRef Medline

18. Voigt, F., Zhang, H., Cui, X. A., Triebold, D., Liu, A. X., Eglinger, J., Lee, E. S., Chao, J. A., and Palazzo, A. F. (2017) Single-molecule quantification of translation-dependent association of mRNAs with the endoplasmic reticulum. Cell Rep. 21, 3740–3753 CrossRef Medline

19. Palade, G. (1975) Intracellular aspects of the process of protein synthesis. Science 189, 347–358 CrossRef Medline

20. Schaletzky, J., and Rapoport, T. A. (2006) Ribosome binding to and dissociation from translocation sites of the endoplasmic reticulum membrane. Mol. Biol. Cell 17, 3860–3869 CrossRef Medline

21. Becker, T., Bhushan, S., Jarasch, A., Armache, J.-P., Funes, S., Jossinet, F., Gumbart, J., Mielke, T., Berninghausen, O., Schulten, K., Westhof, E., Gilmore, R., Mandon, E. C., and Beckmann, R. (2009) Structure of monomeric yeast and mammalian Sec61 complexes interacting with the translating ribosome. Science 326, 1369–1373 CrossRef Medline

22. Prinz, A., Behrens, C., Rapoport, T. A., Hartmann, E., and Kalies, K. (2000) Evolutionarily conserved binding of ribosomes to the translocation channel via the large ribosomal RNA. EMBO J. 19, 1900–1906 CrossRef Medline

23. Pfeffer, S., Burbaum, L., Unverdorben, P., Pech, M., Chen, Y., Zimmermann, R., Beckmann, R., and Förster, F. (2015) Structure of the native Sec61 protein-conducting channel. Nat. Commun. 6, 8403 CrossRef Medline

24. Pfeffer, S., Dudek, J., Gogala, M., Schorr, S., Linzweiler, J., Lang, S., Becker, T., Beckmann, R., Zimmermann, R., and Förster, F. (2014) Structure of the mammalian oligosaccharyl-transferase complex in the native ER protein translocon. Nat. Commun. 5, 3072 CrossRef Medline

25. Voorhees, R. M., Fernández, I. S., Scheres, S. H., and Hegde, R. S. (2014) Structure of the mammalian ribosome-Sec61 complex to 3.4 Å resolution. Cell 157, 1632–1643 CrossRef Medline

26. Potter, M. D., and Nicchitta, C. V. (2000) Ribosome-independent regulation of translocon composition and Sec61 α conformation. J. Biol. Chem. 275, 2037–2045 CrossRef Medline

27. Cui, X. A., Zhang, H., and Palazzo, A. F. (2012) p180 promotes the ribosome-independent localization of a subset of mRNA to the endoplasmic reticulum. PLoS Biol. 10, e1001336 CrossRef Medline

28. Kreibich, G., Freyenstein, C. M., Pereyra, B. N., Ulrich, B. L., and Sabatini, D. D. (1978) Proteins of rough microsomal membranes related to ribosome binding. II. Cross-linking of bound ribosomes to specific membrane proteins exposed at the binding sites. J. Cell Biol. 77, 488–506 CrossRef Medline

29. Harada, Y., Li, H., Li, H., and Lennarz, W. J. (2009) Oligosaccharyltransferase directly binds to ribosome at a location near the translocon-binding site. Proc. Natl. Acad. Sci. U.S.A. 106, 6945–6949 CrossRef Medline

30. Tazawa, S., Unuma, M., Tondokoro, N., Asano, Y., Ohsumi, Y., Ichimura, T., and Sugano, H. (1991) Identification of a membrane protein responsible for ribosome binding in rough microsomal membranes. J. Biochem. 109, 89–98 CrossRef Medline

31. Savitz, A. J., and Meyer, D. I. (1990) Identification of a ribosome receptor in the rough endoplasmic reticulum. Nature 346, 540–544 CrossRef Medline

32. Acosta-Alvear, D., Karagöz, G. E., Fröhlich, F., Li, H., Walther, T. C., and Walter, P. (2018) The unfolded protein response and endoplasmic reticulum protein targeting machineries converge on the stress sensor IRE1. Elife 7, e43036 CrossRef Medline

33. Hsu, J. C.-C., and Nicchitta, C. V. (2018) Proteome expression: the subcellular organisation of protein synthesis. eLS 10.1002/9780470015902.a0005718 CrossRef

34. Potter, M. D., Seiser, R. M., and Nicchitta, C. V. (2001) Ribosome exchange revisited: a mechanism for translation-coupled ribosome detachment from the ER membrane. Trends Cell Biol. 11, 112–115 CrossRef Medline

35. Lingappa, V. R., and Blobel, G. (1980) Early events in the biosynthesis of secretory and membrane proteins: the signal hypothesis. Recent Prog. Horm. Res. 36, 451–475 CrossRef Medline

36. Walter, P., and Johnson, A. E. (1994) Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. Annu. Rev. Cell Biol. 10, 87–119 CrossRef Medline

37. Rapoport, T. A. (1992) Transport of proteins across the endoplasmic reticulum membrane. Science 258, 931–936 CrossRef Medline

38. Roux, K. J., Kim, D. I., Raida, M., and Burke, B. (2012) A promiscuous eukaryotic ribosome binding site. Proc. Natl. Acad. Sci. U.S.A. 109, 1369–1373 CrossRef Medline
mRNA transcriptome localization on the endoplasmic reticulum

42. Deshaies, R. J., Sanders, S. L., Feldheim, D. A., and Schekman, R. (1991) Assembly of yeast Sec proteins involved in translation into the endoplasmic reticulum into a membrane-bound multisubunit complex. Nature 349, 806–808 CrossRef Medline

43. Wild, R., Kowal, J., Eyring, I., Ngwa, E. M., Aebi, M., and Locher, K. P. (2018) Structure of the yeast oligosaccharyltransferase complex gives insight into eukaryotic N-glycosylation. Science 359, 545–550 CrossRef Medline

44. Müller, L., de Escurriaiza, M. D., Lajoie, P., Theis, M., Jung, M., Müller, A., Burgard, C., Greiner, M., Snapp, E. L., Dudek, J., and Zimmermann, R. (2010) Evolutionary gain of function for the ER membrane protein Sec62 from yeast to humans. Mol. Biol. Cell. 21, 691–703 CrossRef Medline

45. Lang, S., Benedix, J., Fedele, S. V., Schorr, S., Schirra, C., Schäuble, N., Jalal, C., Greiner, M., Hassdenteufel, S., Tatzelt, J., Kreutzer, B., Edelmann, L., Krause, E., Rettig, J., Somlo, S., et al. (2012) Different effects of Sec61α, Sec62 and Sec63 depletion on transport of polypeptides into the endoplasmic reticulum of mammalian cells. J. Cell Sci. 125, 1958–1969 CrossRef Medline

46. Ichimura, T., Shindo, Y., Uda, Y., Ohsumi, T., Omata, S., and Sugano, H. (1993) Anti-p34 protein antibodies inhibit ribosome binding to and protein translocation across the rough microsomal membrane. FEBS Lett. 326, 241–245 CrossRef Medline

47. Jagannathan, S., Nwosu, C., and Nicchitta, C. V. (2011) Analyzing mRNA localization to the endoplasmic reticulum via cell fractionation. Methods Mol. Biol. 714, 301–321 CrossRef Medline

48. Stephens, S. B., and Nicchitta, C. V. (2007) In vitro and tissue culture methods for analysis of translation initiation on the endoplasmic reticulum. Methods Enzymol. 431, 47–60 CrossRef Medline

49. Choi-Rhee, E., Schulman, H., and Cronan, J. E. (2004) Promiscuous protein biotinylation by Escherichia coli biotin protein ligase. Protein Sci. 13, 3043–3050 CrossRef Medline

50. Nikonov, A. V., Snapp, E., Lippincott-Schwartz, J., and Kreibich, G. (2002) Active translocon complexes labeled with GFP-Dad1 diffuse slowly as large polyme arrays in the endoplasmic reticulum. J. Cell Biol. 158, 497–506 CrossRef Medline

51. Blobel, G. (2000) Protein targeting. ChemBioChem 1, 86–102 CrossRef Medline

52. Braunger, K., Pfeffer, S., Shrimal, S., Gilmore, R., Berninghausen, O., Mandon, E. C., Becker, T., Förster, F., and Beckmann, R. (2018) Structural basis for coupling protein transport and N-glycosylation at the mammalian endoplasmic reticulum. Science 360, 215–219 CrossRef Medline

53. Wilson, D. N., and Doudna, J. H. (2012) The structure and function of the eukaryotic ribosome. Cold Spring Harb. Perspect. Biol. 4, a011536 CrossRef Medline

54. Zhang, Y., Wollf, T., and Rospert, S. (2013) Interaction of nascent chains with the ribosomal tunnel proteins Rpl4, Rpl17, and Rpl39 of Escherichia coli. J. Biol. Chem. 288, 33697–33707 CrossRef Medline

55. Lin, P. J., Jongsm, C. G., Pool, M. R., and Johnson, A. E. (2011) Polytopic membrane protein folding at L17 in the ribosome tunnel initiates cyclical changes at the translocon. J. Cell Biol. 195, 55–70 CrossRef Medline

56. Cui, K., Coutts, M., Stahl, J., and Sytkowski, A. I. (2000) Novel interaction between the transcription factor CHOP (GADD153) and the ribosomal protein FTE/S3a modulates erythropoiesis. J. Biol. Chem. 275, 7591–7596 CrossRef Medline

57. Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B. M., Strein, C., Davey, N. E., Humphreys, D. T., Preiss, T., Steinmetz, L. M., Kriwzgeld, J., and Hentze, M. W. (2012) Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. Cell 149, 1393–1406 CrossRef Medline

58. Ingolia, N. T., Lareau, L. F., and Weissman, J. S. (2011) Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147, 789–802 CrossRef Medline

59. Schneider-Poetsch, T., Ju, J., Eyler, D. E., Dang, Y., Bhat, S., Merrick, W. C., Green, R., Shen, B., and Liu, J. O. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat. Chem. Biol. 6, 209–217 CrossRef Medline
mRNA transcriptome localization on the endoplasmic reticulum

mRNA-associated granules and bodies. *Mol. Cell* **69**, 517–532.e11

80. Uezu, A., Kanak, D. J., Bradshaw, T. W., Soderblom, E. J., Catavero, C. M., Burette, A. C., Weinberg, R. J., and Soderling, S. H. (2016) Identification of an elaborate complex mediating postsynaptic inhibition. *Science* **353**, 1123–1129

81. Migliaccio, G., Nicchitta, C. V., and Blobel, G. (1992) The signal sequence receptor, unlike the signal recognition particle receptor, is not essential for protein translocation. *J. Cell Biol.* **117**, 15–25

82. Chomczynski, P., and Sacchi, N. (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nat. Protoc.* **1**, 581–585

83. Firat-Karalar, E. N., and Stearns, T. (2015) Probing mammalian centrosome structure using BioID proximity dependent biotinylation. *Methods Cell Biol.* **129**, 153–170

84. Matasova, N. B., Myltseva, S. V., Zenkova, M. A., Graifer, D. M., Vladimirov, S. N., and Karpova, G. G. (1991) Isolation of ribosomal subunits containing intact rRNA from human placenta: estimation of functional activity of 80S ribosomes. *Anal. Biochem.* **198**, 219–223

85. Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120

86. Kim, D., Langmead, B., and Salzberg, S. L. (2015) HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360

87. Anders, S., Pyl, P. T., and Huber, W. (2015) HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169

88. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550

89. Benjamini, Y., and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* **57**, 289–300

90. Almagro Armenteros, J. J., Sønderby, C. K., Sønderby, S. K., Nielsen, H., and Winther, O. (2017) DeepLoc: prediction of protein subcellular localization using deep learning. *Bioinformatics* **33**, 3387–3395