Accurate annotation of human protein-coding small open reading frames

Thomas F. Martinez*, Qian Chu, Cynthia Donaldson, Dan Tan, Maxim N. Shokhirev and Alan Saghatelian

Functional protein-coding small open reading frames (smORFs) are emerging as an important class of genes. However, the number of translated smORFs in the human genome is unclear because proteogenomic methods are not sensitive enough and, as we show, Ribo-seq strategies require additional measures to ensure comprehensive and accurate smORF annotation. Here, we integrate de novo transcriptome assembly and Ribo-seq into an improved workflow that overcomes obstacles with previous methods, to more confidently annotate thousands of smORFs. Evolutionary conservation analyses suggest that hundreds of smORF-encoded microproteins are likely functional. Additionally, many smORFs are regulated during fundamental biological processes, such as cell stress. Peptides derived from smORFs are also detectable on human leukocyte antigen complexes, revealing smORFs as a source of antigens. Thus, by including additional validation into our smORF annotation workflow, we accurately identify thousands of unannotated translated smORFs that will provide a rich pool of unexplored, functional human genes.

Annotation of open reading frames (ORFs) from genome sequencing was initially carried out by locating in-frame start (AUG) and stop codons. This approach resulted in unreasonably large numbers of ORFs smaller than 100 codons, called smORFs. A length cutoff was then introduced to remove smORFs, which were largely presumed to be meaningless random occurrences. With the advent of more sensitive detection methods, functional proteins encoded by smORFs, dubbed microproteins, have been characterized with more regularity. In fruit flies, talATU was shown to encode three 11- and one 32-amino acid microproteins that control proper physiological development. This example, and others, highlighted the importance of investigating smORFs, and paved the way for work in higher organisms. Recently, several mammalian microproteins have been characterized, with fundamental roles including DNA repair, mitochondrial function, RNA regulation and muscle development. These studies demonstrated that genomes contain many functional smORFs and, therefore, annotating all protein-coding smORFs is important.

Advances in proteomics and next-generation sequencing (NGS) technologies provided the tools necessary to identify protein-coding smORFs. For example, the integration of RNA-seq and proteomics approaches identified hundreds of unannotated microproteins in human cell lines. While proteomics provides evidence that a smORF produces a microprotein of sufficient abundance for detection, it is limited in sensitivity and some microproteins do not have suitable tryptic peptides. With the development of ribosome profiling (Ribo-seq), NGS can be utilized to identify ORFs that are undergoing active translation, with high sensitivity and accuracy, by revealing the position of elongating ribosomes throughout the transcriptome. Ribo-seq has been applied successfully to smORF discovery in fruit flies and zebra fish, identifying hundreds of previously unknown translated smORFs, which is significantly more than were detected by mass spectrometry in these organisms.

Ribo-seq has also been used more recently to annotate protein-coding smORFs in human cell lines and tissues. SmProt and sORFs.org are two prominent smORF databases, containing >17,000 and >500,000 unique Ribo-seq-predicted human protein-coding smORFs, respectively. However, this order of magnitude difference, despite analyzing many of the same datasets, raised concerns, as accurate smORF annotations are critical for downstream biological studies. SmProt and sORFs.org employ different strategies for identifying and filtering protein-coding smORFs, which may contribute to the size disparity. Another possible contributor is that unannotated smORFs might be less reliably called translated rather than annotated ORFs using Ribo-seq due to their low relative abundance, inherent small size or other distinguishing properties. Thus, major questions about smORFs remain. (1) Is Ribo-seq as robust at identifying translated unannotated smORFs as annotated ORFs? (2) How many bona fide protein-coding smORFs are in the human genome? (3) Is there evidence that protein-coding smORFs are regulated similarly to annotated genes? To answer these questions, we developed a top-down workflow that combines de novo transcriptome assembly and multiple Ribo-seq experiments to rigorously annotate protein-coding smORFs.

We found that while detection of annotated ORFs is robust, smORF detection is noisier. Application of this workflow in HEK293T, HeLa-S3 and K562 cells uncovered >2,500 confidently annotated protein-coding smORFs—our gold standard set—and >7,500 smORFs in total. We also demonstrated that while smORF-encoded microproteins have distinguishing properties from annotated proteins, their expression is similarly regulated during cell stress, and they are also presented as cell-surface antigens. These results dramatically increase the coding potential of the genome and provide several strategies for finding potentially functional smORFs.

Results

Overview of top-down smORF annotation workflow. Ribo-seq maps the position of elongating ribosomes throughout the transcriptome by first footprinting with RNase I (Fig. 1). The resulting...
Fig. 1 | Outline of top-down smORF annotation workflow. RNA-seq and Ribo-seq datasets were collected for HEK293T, HeLa-S3 and K562 cell lines and utilized for the prediction of unannotated translated smORFs. RNA-seq reads were assembled into a transcriptome using Cufflinks. The assembled transcriptome for each cell line was then three-frame translated in silico to create a database of all possible ORFs. In parallel, multiple biological replicates of Ribo-seq data were also collected for each cell line and utilized to assess translation of all smORFs in the accompanying three-frame database. For each replicate, RibORF was used to define the A-site position of each RPF and then score each smORF for translation. Those smORFs that passed RibORF scoring, did not overlap with annotated ORFs and lacked similarity to RefSeq-annotated proteins were retained. Shown at the bottom are examples of a smORF passing RibORF scoring with high coverage and in-frame ribosome A-site reads (frame 1) and a smORF failing RibORF scoring due to poor read coverage.
28–29 nucleotide (nt) ribosome-protected messenger RNA fragments (RPFs) are then sequenced and aligned to the transcriptome. Typically, Ribo-seq reads are mapped onto reference transcriptome databases, such as RefSeq or Ensembl, which are not representative of every cell type. Our top-down workflow utilizes transcripts obtained by de novo assembly of RNA-seq data. This approach identified entirely new transcripts as well as isoforms of annotated transcripts, allowing for more comprehensive smORF discovery. We then define ORFs across all three reading frames of the de novo-assembled transcriptome to generate an ORF database that includes smORFs.

After obtaining Ribo-seq data, we scored all ORFs in the database for translation using RibORF (Fig. 1), a support vector machine-based classifier of translation\(^ {22} \). RibORF uses the fraction of RPF reads aligned in-frame with the candidate ORF to calculate the overall probability of translation, which depends on the resolution of the dataset. Sub-codon or high-resolution Ribo-seq datasets can display >70% of RPFs aligned in-frame with annotated coding sequences (CDS)\(^ {23} \) by metagene analysis, enabling more accurate identification of unannotated protein-coding smORFs. RibORF also scores the uniformity and distribution of RPF reads over the entire ORF, to avoid possible artifacts\(^ {22} \).

Following RibORF scoring, the list of predicted translated ORFs was filtered to remove ORFs less than 6 codons, which are not amenable to detection by mass spectrometry, and greater than 150 codons, as unannotated protein-coding smORFs larger than 100 codons have been discovered\(^ {21} \). Next, translated smORFs found to overlap with annotated CDS regions in the UCSC database were removed to filter out both annotated genes and out-of-frame overlappers. Finally, encoded microproteins were analyzed for similarity to human RefSeq proteins by BLASTp. Only low-scoring hits were retained, removing likely pseudogenes and any additional annotated genes. The remaining hits constitute the set of unannotated microprotein-encoding smORFs (Fig. 1).

Annotating protein-coding smORFs in HEK293T cells. We first tested our workflow in HEK293T cells, which we previously identified dozens of microproteins in by proteomics\(^ {15} \). Ribosome footprints were initially prepared using a protocol that afforded high-resolution data in HEK293 cells\(^ {2} \). However, only ~50% of reads aligned in-frame by metagene analysis, and RPF lengths peaked at 31 nt (Fig. 2a). While this resolution is comparable to several published datasets (Supplementary Fig. 1), we collected higher resolution datasets as well, to ensure identification of translated smORFs that require greater accuracy. To gain finer control over nuclease digestion, we followed a reported strategy that normalizes the amount of nuclease added to the RNA concentration\(^ {23} \). We generated two additional HEK293T Ribo-seq datasets, with ~60% and >70% of reads in-frame by metagene analysis and RPF lengths that peaked at 30 and 28 nt, respectively (Fig. 2a). Given that published datasets show a wide range of resolutions (Supplementary Fig. 1), we carried all three datasets forward for protein-coding smORF prediction.

Several previous studies combined reads from multiple Ribo-seq experiments to increase the sensitivity of translation scoring\(^ {22–24} \). However, this strategy can also allow for more false positives when the same thresholds are applied, due to reads accumulating on an ORF because of nonproductive ribosomal binding or noise inherent to the Ribo-seq protocol\(^ {24} \). Additionally, combining experiments does not allow one to assess the reproducibility of translation predictions, which is critical in other NGS-based assays\(^ {22} \). When analyzed separately, smORFs scored as translated in every experiment regardless of noise and sequencing depth are more confidently protein-coding than those found in a single experiment, and also allow one to observe how differences in RPF preparation affect translation scoring. Therefore, to improve the confidence of smORF translation prediction, we analyzed each Ribo-seq experiment separately.

To confirm the quality of our HEK293T Ribo-seq datasets and determine the noise level for bona fide genes, we used RibORF to score RefSeq genes. Despite differences in resolution and sequencing depth, we observed high overlap among the 9,644 canonical genes called translated, with 74% found in all three experiments (Fig. 2b). For smORFs, however, we found that these differences had a strong influence on the total number called translated (Fig. 2c). We identified 1,913, 2,401 and 572 predicted translated smORFs, with 117 smORFs called translated in every experiment and 895 smORFs in at least two experiments. Interestingly, 606 smORFs were found in both lower resolution datasets but not in the high-resolution dataset. Thus, translation prediction is noisier for smORFs than for annotated ORFs, but the analysis of several Ribo-seq experiments can improve confidence. The set of reproducibly detected smORFs is also greater than the 24 microproteins that we identified in HEK293T cells by proteomics\(^ {15,18} \), highlighting the value of Ribo-seq for smORF discovery. Ribo-seq data validated nine of our proteomics-detected smORFs (Supplementary Data 1), with the others missing due to overlap with annotated genes or insufficient read coverage.

Endoplasmic reticulum stress-regulated smORFs. Having identified thousands of unannotated protein-coding smORFs, we next searched for evidence of their regulation as a means to uncover possible biological roles. We chose to look for expression changes induced by endoplasmic reticulum (ER) stress, which leads to the accumulation of unfolded and misfolded proteins, and triggers a well-characterized signaling cascade dubbed the unfolded protein response (UPR)\(^ {30} \). To induce ER stress, HEK293T cells were treated with either thapsigargin (TG) or tunicamycin (TM), and RNA-seq and Ribo-seq data were collected for each sample (Supplementary Figs. 2a and 3). Applying our workflow, we identified 666 additional predicted translated smORFs, increasing the total to 4,540 in HEK293T cells (Supplementary Data 1). Comparing TG- and TM-induced activation of the UPR, HSPA1, HYOU1, DDIT3 and other known UPR genes were upregulated\(^ {31} \) (Fig. 3a and Supplementary Data 2). Gene ontology analysis also revealed enrichment in UPR and cell stress-related genes (Supplementary Data 2).

We then analyzed smORFs for transcriptional regulation under ER stress, focusing on reproducibly detected smORFs. TG and TM induced significant mRNA expression changes in 43 and 7 smORFs, respectively (Fig. 3a), suggesting that the encoded microproteins might function in the UPR. For instance, one upregulated smORF, UPR-smORF1, is found on a de novo-assembled transcript isoform of asparagine synthase pseudogene 1 (ASNSP1) (Fig. 3b and Supplementary Data 2). While ASNSP1 is a predicted pseudogene, translated pseudogenes are being reassessed for functional importance\(^ {32} \). In addition, asparagine synthase (ASNS) is a known UPR target gene\(^ {32} \), supporting the possibility that the UPR-smORF1 microprotein might have a role in the UPR.

Several UPR pathway genes have been shown to be translationally regulated during ER stress\(^ {33–34} \). Therefore, we sought to identify any translationally regulated smORFs. Translational regulation was monitored by assessing changes in translational efficiency (TE) using Xtail\(^ {35} \), which quantifies the changes in RPF densities relative to mRNA expression levels using Ribo-seq and RNA-seq data, respectively. Both TG and TM induced higher TE for ATF4, IFRD1 and SEC61G, which are known to be regulated during ER stress\(^ {36–38} \), as well as many other genes (Supplementary Fig. 4a,b,c and Supplementary Data 2). Analysis of smORFs revealed a robust change in TE for a single smORF located on SNHG8, dubbed UPR-smORF2 (Fig. 3c). Increased TE for UPR-smORF2 is clearly visualized by comparing the Ribo-seq and RNA-seq read coverage plots for SNHG8 (Fig. 3d), which show an increase in ribosome occupancy and little change.
in transcript levels between vehicle- and TG-treated cells. In addition, the TE of an annotated but uncharacterized smORF, c14orf119, significantly decreased in response to TG.

**Annotation of smORFs in additional cell lines.** To determine whether the smORFs identified in HEK293T are unique, and to test the generality of our observations, we profiled additional cell lines for protein-coding smORFs. Because they differ from HEK293T in their tissue of origin, we selected the chronic myeloid leukemia-derived cell line K562 and the cervical cancer-derived HeLa-S3 cell line. Both of these cell lines are also included in ENCODE, providing a wealth of high-quality genomic, transcriptomic and functional data available for follow-up analyses.

As with HEK293T, HeLa-S3 cell lysates were digested using different conditions to maximize the number and accuracy of the smORFs identified. Metagene analysis showed a range of resolutions across the four datasets collected, from ~50–70% reads in-frame (Supplementary Figs. 3b and 5). Altogether, 2,614 unannotated smORFs were called translated, with 777 smORFs found in at least two experiments (Supplementary Data 1). Next, we collected three Ribo-seq datasets from K562 using a range of digestion conditions. All digestion conditions tested in K562 resulted in >75%
reads in-frame by metagene analysis (Supplementary Fig. 6). However, K562 HiRes3 displayed a broader footprint length distribution (Supplementary Fig. 2c). In total, 2,464 predicted protein-coding smORFs were identified in K562 cells, with 542 smORFs found in at least two experiments (Supplementary Data 1). Across the three cell lines profiled, we identified 7,554 predicted protein-coding smORFs. The majority of these smORFs are only identified in a single experiment, but there are thousands of smORFs that overlap between cell lines or are found in multiple experiments from a single cell line. In total, 483 smORFs were detected in all three cell lines, 1,581 in at least two cell lines and 2,689 in at least two experiments across any cell line (Fig. 4a,b). We define this last set of smORFs as our gold standard protein-coding smORF annotation, given their reproducibility. These results reveal that smORFs, similarly to larger annotated genes, can be ubiquitous and cell-type specific. Notably, we also observed that smORFs called translated in two or more cell lines are more likely to utilize an AUG initiation codon than smORFs found in only one cell line (Fig. 4a), which supports their robust detection across different cell types.

Next, we quantified the abundance of smORF-containing transcripts to determine whether smORFs called translated in only a single experiment are relatively less expressed. We found that the median transcript fragments per kilobase per million reads mapped (FPKM) values are significantly greater for smORFs called translated in multiple experiments than for singly identified smORFs (Supplementary Fig. 7). These results suggest that the ability to
reproducibly detect translated smORFs by Ribo-seq may be limited in part due to transcript abundance.

**Protein-coding smORFs on annotated transcripts.** Over half of all predicted translated smORFs are located on RefSeq transcripts. The majority of smORFs are found within the 5′ untranslated region (UTR) of known genes (Supplementary Fig. 8), including ~76% of predicted translated smORFs identified in all three cell lines. These 5′ UTR smORFs are also called upstream open reading frames (uORFs), and often regulate translation of the downstream CDS.
through engagement with the ribosome\(^{49}\). While the microprotein products of uORFs are often assumed to be nonfunctional, there are examples with characterized functions, such as the 70-amino acid *MIEF1* uORF microprotein\(^{14,44}\). We identified 597 uORFs containing >50 codons that are candidates for encoding functional microproteins (Supplementary Data 1).

Beyond uORFs, a small portion of predicted protein-coding smORFs were found within the 3′ UTR, on antisense transcripts, and on noncoding RNAs (ncRNA). Notably, 623 translated smORFs are located on RefSeq ncRNAs, and several more on UCSC ncRNAs, and many are high-confidence identifications found in several experiments. For instance, translated smORFs on the ncRNAs, *BC013229* and *LOC100287015* (Supplementary Fig. 9a,b), were identified in every HeLa-S3 dataset. We also observed ncRNAs containing multiple protein-coding smORFs, such as *LINC00534*, which contains two smORFs in different reading frames (Supplementary Fig. 9c). Some ncRNAs even contained more than two predicted translated smORFs, such as the colon cancer-related gene *CCAT1*, which contains two confidently identified smORFs and several more called translated only once (Supplementary Fig. 9d and Supplementary Data 1).

**Analyzing microprotein properties.** We next sought to determine distinguishing properties of microproteins from annotated proteins. First, the median length of encoded microproteins is 32 amino acids (Fig. 4c, red line), whereas the median human protein length in the Pfam database is 416 amino acids\(^{43}\). The frequency distribution of microprotein lengths can be fit by a decay curve that has a slower decay than expected for randomly occurring microproteins, based on an ~5% chance of encountering a stop codon\(^7\) (Fig. 4c). Thus, predicted protein-coding smORFs occur at a higher frequency than expected by chance alone. In addition, comparing microprotein amino acid usage to that of annotated proteins revealed a clear difference in several amino acid frequencies, including increased usage of alanine, glycine, proline and arginine, as well as depletion of aspartic acid, glutamic acid, isoleucine, lysine and tyrosine (Fig. 4d). We also analyzed microproteins for common structural features, including transmembrane helices and conserved protein domains. Only 48 predicted transmembrane helix domains (Supplementary Data 3), and another 17 are predicted to contain conserved protein domains.

Given that uORFs might generally behave as regulators of downstream translation, we also checked whether their encoded microproteins differ from those of non-uORFs. We found that the median length of uORF microproteins is shorter than for non-uORF microproteins, at 24 versus 43 amino acids, respectively (Supplementary Fig. 10a,b). However, uORFs and non-uORFs show little difference in amino acid usage (Supplementary Fig. 10c). We also compared the transcript abundances and ribosome densities for uORFs versus non-uORFs (Supplementary Fig. 10d,e). These results are consistent with uORFs sharing their transcripts with relatively well-expressed annotated ORFs and their regulation of downstream translation.

**Evidence of smORF conservation.** Based on functionally characterized smORFs\(^{41,42,43,44}\), we hypothesized that some microproteins would show sequence conservation across other mammalian species. We first employed PhyloCSF, which uses a multispecies nucleotide alignment to examine sequences for signatures of conserved coding regions\(^{41}\). At least one exon with a positive average PhyloCSF score was found in 432 smORFs (Supplementary Data 1), such as the smORF within the 5′ UTR of *FJX1* (Fig. 4e). We also searched for sequence similarities across other species using tBLASTn and BLASTp as evidence for possible protein conservation. Using tBLASTn, 4,687 microproteins were found to have high similarity to translated RNA sequences from at least one other species, including 273 to mouse sequences (Supplementary Data 1). Additionally, 476 microproteins with high similarity to known and predicted proteins were found in other species using tBLASTn and BLASTp. In many instances, clear sequence similarity was observed across several species using tBLASTn and BLASTp, despite having negative PhyloCSF scores (Fig. 4f,g). These data suggest that many microproteins show evidence of conservation, and therefore are likely to have cellular and physiological functions.

**Identifying smORF translation initiation sites.** Approximately 40% of the predicted protein-coding smORFs lack an in-frame canonical AUG start codon (Fig. 4a), making their translation initiation sites difficult to identify. Through treatment with initiation-specific inhibitors, such as harringtonine (Harr) and lactimidomycin (LTM)\(^{42}\), one can use Ribo-seq to identify translation initiation sites. For example, Harr treatment induced RPF accumulation centered on the first AUG start codon in a *METTL3* uORF (Supplementary Fig. 11a). Start-site inhibitors also helped identify alternative initiation codons. LTM treatment enriched RPF coverage over the near-cognate start codon UUG in a *TMEM33* uORF (Supplementary Fig. 11b), supporting its translation despite the lack of an in-frame AUG start codon.

These inhibitors were also helpful in identifying the predominant codons for translation initiation when multiple canonical or near-cognate start codons were present. For example, there are three in-frame AUG codons within a uORF on *GTF2H1*. Surprisingly, Harr treatment induced the highest RPF accumulation on the third AUG codon, with only a small peak present over the first AUG (Supplementary Fig. 11c), suggesting that both a long and predominant short form of the microprotein are made. Similarly, we observed mixed start site usage for the uORF on *FBXO9*, with translation initiation peaks on a CUG codon and a downstream AUG codon (Supplementary Fig. 11d). Interestingly, no initiation peak was observed over the most upstream in-frame AUG codon.

**Protein-coding smORFs on unannotated transcripts.** By including de novo transcriptome assembly, we were able to identify a large portion of predicted protein-coding smORFs on transcripts that are missing from the RefSeq assembly. For example, we observed a 5′ extension of *c6orf62*, which contains a translated smORF (Supplementary Fig. 12a). Other examples include entirely unannotated exons, such as the smORF-containing *EYA4* isoform found specifically in HeLa-S3 samples (Supplementary Fig. 12b) and the *GGPS1* isoform with an alternative 5′ UTR containing a smORF (Supplementary Fig. 12c).

Several predicted protein-coding smORFs were also found on transcripts that do not overlap with any annotated gene, and many of these unannotated transcripts are cell-type specific (Fig. 5a–c). BLAST sequence analysis can help to identify the function of these unannotated genes. For instance, the HEK293T-specific smORF-encoded microprotein in Fig. 5a shows high similarity to a sequence on the X-linked reproductive homeobox (rhox) pseudogene *RHOXF1P1*, as well as to two predicted rhox-like X-linked homeobox genes in other mammals (Fig. 5d and Supplementary Data 1). Moreover, this X-linked homeobox candidate is located within 90 kilobases (kb) of the rhox gene cluster and within 20 kb of *RHOXF1P1*. Given that there are 33 known rhox genes in mouse but only three in humans\(^{45}\), it is possible that this smORF is a missing rhox family gene.

**Detection of microprotein peptides on HLA-I complexes.** While Ribo-seq is effective for identifying translated smORFs, it cannot determine whether the encoded microproteins are sufficiently long lived to be functional. Mass spectrometry provides direct evidence of proteins that accumulate to a concentration above the limit of detection, offering important complementary data. We therefore
complex using a fluorescence-based competition assay (Fig. 6d) and also verified binding of three microprotein peptides to the HLA-I regardless of the number of times detected by Ribo-seq (Fig. 6c). We re-analyzed published proteomics datasets to validate some of the Ribo-seq predicted translated smORFs. Proteomic analysis of immunoprecipitated HLA-I complexes has been used to identify antigenic peptides from annotated genes. We reasoned that HLA-I immunoprecipitation serves as an ideal enrichment step to enhance antigenic peptides from annotated genes. We intended incorporated Ribo-seq data of varying resolution into our workflow differs in several key ways from the SmProt\(^2\) and sORFs.org\(^2\) databases, which improves the quality of our smORF annotations. Second, we define smORFs by the most upstream in-frame AUG start codon or stop codon and protein sequence, as well as predicted X-linked homeobox genes in C. capucinus and S. boliviensis.

**Fig. 5 | Protein-coding smORFs identified on unannotated transcripts.** a–c Predicted protein-coding smORFs were identified on unannotated de novo-assembled transcripts that had no overlap with annotated genes. Examples shown are specific to HEK293T a, HeLa-S3 b and K562 c. The top plot shows RNA coverage at the genomic level with the exon model of the Cufflinks-assembled transcript shown above. Black boxes represent the exons, connecting lines represent the introns and the strand orientation is noted by the arrowhead. The middle A-site plot shows the Ribo-seq coverage at the transcript level, with reads colored by frame. The smORF coordinates are shown in the top corner. The smORFs in a, b and c are in frame 1, while the smORF in d is in frame 2.

**Comparison with other smORF databases.** Our Ribo-seq-based workflow differs in several key ways from the SmProt\(^3\) and sORFs.org\(^2\) databases, which improve the quality of our smORF annotations. First, we intentionally incorporated Ribo-seq data of varying resolution and demonstrate that it affects smORF translation prediction. Neither SmProt nor sORFs.org show the metagene analyses for the published datasets utilized in their workflows, making it impossible to tell whether the underlying data used are of sufficient quality for smORF annotation. Second, we define smORFs by the most upstream in-frame AUG start codon or stop codon and protein sequence, as well as predicted X-linked homeobox genes in C. capucinus and S. boliviensis.
smORFs that overlap with annotated ORFs, which we leave out due to the increased likelihood of being scored inaccurately with low-resolution data. Another key difference is that sORFs.org uses its own noise-filtering algorithm, which does not incorporate three-nucleotide periodicity, leading to many smORFs called translated despite poor Ribo-seq evidence. SmProt utilizes RiboTaper for its
translation predictions, which incorporates three-nucleotide periodicity similarly to RibORF and several other translation-scoring software packages. Lastly, sORFs.org contains many smORF entries that overlap in-frame with annotated ORFs, and which cannot be separated as unique translation products by Ribo-seq.

As a result, our gold standard database contains fewer unique annotated smORFs than SmProt and sORFs.org. Despite having more protein-coding smORF entries, both these other databases miss a substantial number of smORFs that are annotated in our datasets. The sORFs.org database contains 3,269 predicted translated smORFs in common with our annotations, only 1,574 of which overlap with our gold standard set (Supplementary Data 1). Similarly, SmProt shares just 1,169 Ribo-seq annotated smORFs in common with our database, 798 of which overlap with our gold standard set. SmProt and sORFs.org are also missing many smORFs for which we identified peptides in the HLA-I proteomics data, including only 217 and 128 out of 320 smORFs, respectively. Thus, our database retains high-confidence annotations without incorporating as many likely false positives. Still, both SmProt and sORFs.org include more Ribo-seq datasets from additional cell lines, and thus likely include bona fide protein-coding smORFs that were not found in our datasets.

**Discussion**

This study serves three key purposes: the development of a reliable workflow for smORF annotation, the curation of a human protein-coding smORF database and the demonstration of strategies for finding smORFs related to pathways of interest. Utilizing our workflow, we were able to rigorously annotate thousands of protein-coding smORFs in three human cell lines. By analyzing individual experiments, we showed that predicting smORF translation from Ribo-seq data is noisier than for annotated genes. Differences in Ribo-seq resolution, sequencing library construction, sequencing depth, as well as biological variations such as passage number and cell density can affect smORF translation analysis. However, given that annotated ORFs showed much greater overlap between the same experiments, it is most likely that overall lower transcription and translation levels explain why smORFs are more difficult to detect reproducibly.

We also show that it is beneficial to use a range of RNase I digestion conditions to annotate smORFs, as there are several hundred reproducibly detected smORFs that were only identified in lower or higher resolution datasets. Based on these results, we suggest that there is an optimal range of digestion conditions for identifying translated smORFs, below which translation predictions have low accuracy and above which there is overall reduced RPF coverage. This latter point is supported by previous studies, which showed that monosome stability is particularly sensitive to digestion by RNase I compared with other RNases in some mammalian cell lines and tissues. Importantly, we also demonstrate that de novo transcriptome assembly is necessary for comprehensive smORF annotation.

For many smORFs, these data provide the first evidence of translation. Therefore, we propose using our higher confidence gold standard set of >2,500 smORFs called translated in multiple experiments for follow-up functional studies. For a smaller library, one can use the even higher confidence set of smORFs found in multiple cell lines, which require both transcript assembly and sufficient Ribo-seq evidence in each cell line. Supporting their higher confidence, our gold standard set is enriched among smORFs validated in the HLA-I proteomics data, although this could also suggest that microproteins found in multiple cell lines are more likely to have peptides presented on HLA-I complexes. Notwithstanding their lack of reproducibility, smORFs identified in a single experiment are worth including in large-scale studies, as many just failed the stringent RibORF scoring filter in other experiments and might pass with higher sequencing depth or in an additional replicate. In support of their inclusion, peptides from singly identified smORFs were also validated in the HLA-I proteomics data, and over 1,800 overlapped with the sORFs.org or SmProt databases.

Beyond reproducibility, useful methods for uncovering biologically functional smORFs include identifying those that are regulated, bound to protein complexes or evolutionarily conserved. For example, smORFs that are regulated during ER stress, such as UPR-smORF1 and UPR-smORF2, might have functions in the UPR. Similarly, microprotein peptides presented on HLA-I complexes may be immunogenic or serve as useful biomarkers. Functional inferences can also be drawn from microprotein sequence similarity, such as the potential X-linked homeobox gene in HEK293T cells. Having identified thousands of smORFs, additional biological data can easily be mined to help elucidate their roles.

While our data represent a important step in comprehensive protein-coding smORF annotation, we expect future studies to find additional smORFs. First, these numbers are an underestimation, because we chose to exclude smORFs that overlap with annotated ORFs in our analyses, although such smORFs are known. By definition, overlapping smORFs have RPF reads aligned out-of-frame relative to another ORF, which limits the scoring of both. Our highest resolution datasets may be suitable for identifying abundant overlappers; however, we expect to find a large number of artifacts using our lower resolution datasets due to the higher percentage of noisy out-of-frame reads. Second, we utilized ENCODE cell lines, which are valuable but likely different from primary cells or tissues. Finally, improvements to transcript assembly through long-read sequencing, small RNA library construction and to computational methods for short-read alignment and analysis of Ribo-seq for translation will be critical for complete annotation of protein-coding smORFs. There are currently several newer translation-scoring software packages that could help identify additional smORFs that are missed by RibORF.

Given the number of protein-coding smORFs annotated, their diversity of amino acid composition and their cell-type specificity, we anticipate smORFs being involved in all facets of biology. In addition, new insights into translational regulation can be gained by studying polycistronic RNAs and how multiple start sites are employed for the same reading frame. These results also add to the growing evidence that some ncRNAs can operate as both a functional molecule and a coding template. In summary, smORFs offer a rich opportunity for uncovering new biology, and in the future, perhaps, a new avenue for therapeutic discovery.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41589-019-0425-0.

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Methods

Cell culture. HeLa-S3 cells (CCL-2.2) were purchased from American Type Culture Collection. HEK293T cells (HCL4517) were purchased from GE Life Sciences. K562 cells (CD141047) were purchased from MilliporeSigma. HEK293T and HeLa-S3 cells were maintained in DMEM (Corning, catalog no. 31980-020) supplemented with 10% FBS (Corning, catalog no. 35-010-CV). K562 cells were maintained in RPMI 1640 (Corning, catalog no. 10-040-CV) supplemented with 10% FBS. All cells were maintained at 37°C with 5% CO2.

Paired-end RNA-seq and de novo transcriptome assembly. The HEK293T Cufflinks-assembled transcriptome was generated previously19, and used to create the ORF database for scoring translation with RibORF. For HELa-S3 and K562, total RNA was collected and purified from two biological replicates using an RNaseasy kit (Qiagen) with genomic DNA elution columns. For each cell line, two separate complementary DNA libraries were prepared for each replicate: one using the TruSeq Ribo Profiling RNA kit (Illumina) and the other using Total RNA kit (Illumina). This allowed for representation from poly(A)-tailed mRNA and non-poly(A) RNAs in the transcriptome assembly. Paired-end 125 or 150 base reads were collected for all four libraries on a single lane of an Illumina HiSeq 2500 or NextSeq 500, respectively. At least 250 million reads were generated for each cell line. Aligned reads were assembled into transcripts by Cufflinks using rPKM scores to identify the most distal in-frame stop codon for every stop codon. By including only the first position of the stop codon in the ORF definition, we limited the scoring penalty that frequently occurs due to the higher frequency of out-of-frame reads. A previous study dealt with the extreme nature of translation termination peaks by excluding the stop codon altogether from scoring21, while others include the entire stop codon and do not handle it differently22. While the majority of smORFs called translated do not change whether the stop codon is included or not, our strategy results in the highest number of predicted protein-coding smORFs and offers the best overlap with each alternative option across all different levels of overall RNA-seq resolution tested (Supplementary Fig. 14).

Ribosome footprinting. Preparation of ribosome footprints for Ribo-seq experiments was performed as described16 with some modifications. For all ribosome footprinting experiments, adherent cells were grown to about 80% confluence in 10-cm or 15-cm diameter tissue culture dishes, and suspension cells were grown to a density of approximately 500,000 cells per ml. Cells were washed with 5 ml ice-cold PBS with 100µg/ml cycloheximide (CHX) added. Immediately after removing PBS, 400 µl of ice-cold lysis buffer (20mM Tris–HCL, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% Triton X-100, with 1 mM DTT, 25 µM Turbo DNase (Thermo Fisher, catalog no. AM2238) and 100 µg/ml CHX added fresh) was dripped onto the plate or added to the cell pellet. Cells were incubated on ice in lysis buffer for 10 min with periodic vortexing and pipetting to disperse the cells. The lysate was then clarified by centrifugation at 15,000 g for 10 min. Cell lysates were flash-frozen in liquid nitrogen and stored at −80°C for up to 5 days before ribosome footprinting. For experiments profiling translation initiation, the same procedure was followed except for the addition of either 2µg/ml harringtonine (Abcam, catalog no. ab141941) for 2 min or 20µg/ml lactacystin (MilliporeSigma, catalog no. 506291) for 30 min to media, before PBS wash and lysis. A variety of dissociation conditions were tested in this study and are summarized in Supplementary Data 1. Briefly, RNA digestions using 250 U RNase I (Thermo Fisher, catalog no. AM2294) per 100 µl lysate was used in the low-resolution 293T and HeLa-S3 experiments. For high-resolution experiments, 15–30 U TruSeq Nuclease (Illumina) was used to digest 30–60 ng total RNA in up to 300µl lysate. Digestion reactions were run for 45–60 min at room temperature and quenched with 100–200 U Supersafe-In RNase I inhibitor (Thermo Fisher, catalog no. AM2694) on ice. Following digestion, RPFs were purified from small RNA fragments using MicroSpin S-400 HR columns (GE Life Sciences) according to the TruSeq Ribo Profile kit (Illumina). Low-resolution experiments were cleaned up with ZymoClean DNA Clean & Concentrator-5 (Zymo), while high-resolution experiments were purified by acid phenol:chloroform extraction followed by isopropanol precipitation. Ribosomal RNAs were depleted from RPF fragments by Ribo-Zero Mammalian kit (Illumina) following the manufacturer’s protocol. cDNA sequencing libraries were then prepared using the TruSeq Ribo Profile kit (Illumina) following the manufacturer’s protocol. Single-end 50-base reads were collected on an Illumina Hiseq4000 with no more than four samples sequenced on a single lane. Each Ribo-seq experiment was prepared from a different biological replicate, except for K562 HiRes1 and 2, which were prepared from the same lysate using different digestion conditions. For K562 HiRes3, CHX was added to the media before pelleting cells and washing with PBS.

Ribo-seq and short-read RNA-seq read processing. Ribo-seq and accompanying short fragment total RNA-seq reads were first trimmed of excess 3’ adapter sequences, as in Calviello et al.20 using the FASTX toolkit. Trimmed Ribo-seq reads aligning to transfer RNA and ribosomal RNA sequences were then removed using STAR v2.5.2b (ref.21) as in Wang et al.20. Next, the remaining Ribo-seq reads were aligned to the UCSC hg19 human genome assembly containing chromosomal 1–22, X and Y, with the hg19 RefGene transcript annotation using STAR. Up to two mismatches were allowed during alignment, keeping only uniquely mapped reads. Ribo-seq and RNA-seq alignment checks were performed for overall quality using the CollectRnaSeqMetrics script from the Picard Tools software suite.

RibORF scoring. Following Ribo-seq read processing and quality control, the RibORF software package22 was used to score individual ORFs for translation. First, metagene analysis was conducted using coding genes from the hg19 RefGene annotation included with RibORF. Metagene analysis is run for individual processed read lengths ranging from 25–34 nt. Using the metagene plots, the offset shift needed to align the 5’-most position with the A-site, or +3 position, for each read length was assessed. Next, the entire Ribo-seq alignment was corrected by the offset shift for each length. For high-resolution data, reads ranging from 25 to 30 nt in length were included depending on the sample’s footprint length distribution. For lower resolution data, reads ranging from 28 to 35 nt were included. The offset-corrected read alignments were used for scoring individual ORFs as translated. Following the suggestions of the RibORF developers, only ORFs with RibORF scores ≥0.7 and at least ten reads mapped to the ORF were considered translated in each individual Ribo-seq dataset. Each Ribo-seq dataset was analyzed individually for translated smORF predictions. RNA coverage and Ribo-seq A-site plots for individual smORFs were plotted using R scripts.

Defining ORFs. RibORF does not define boundaries of putative ORFs based on Ribo-seq coverage and thus requires a user-generated list of candidate ORFs. Generation of ORF databases from the de novo-assembled transcriptome of each cell line was done using a custom java script, GTNfastA (Supplementary Data 4). For the de novo-assembled transcriptome of each cell line, ORFs were defined by identifying the most distal in-frame upstream AUG start codon for every stop codon across all three reading frames. Because Ribo-seq evidence is expected to occur solely within a putative ORF, it is important to limit ORFs to AUG start codons, which are most likely to be initiation sites on the basis of the scanning model of translation, when available, instead of beginning at upstream stops. However, if no AUG start codon is found, the ORF was defined from stop codon to the first position of non-AUG-initiation. The resulting millions of ORFs were then assembled into a database containing the exon coordinates for each ORF in refFlat format. In Ribo-seq datasets, translation termination peaks are often overrepresented and have a higher fraction of reads aligned to the second position (out-of-frame) compared with non-stop codons, as observed by metagene analysis (Fig. 2a). Therefore, for RibORF scoring, only the first position of the stop codon was included in the ORF definition. For lower resolution data, reads ranging from 28 to 35 nt were included. The offset-corrected read alignments were used for scoring individual protein-coding smORFs and offers the best overlap with each alternative option across all different levels of overall Ribo-seq resolution tested (Supplementary Fig. 14).

Differential translation analysis. Differential translation analysis was conducted using the R package Xtail v1.1.5 (ref.23). First, HTSeq-count38 in intersection-strict mode was used to calculate total RNA read counts for hg19 RefGene annotations. For smORFs, HTSeq-count was run in union mode and allowed for non-unique reads to be counted. RPF read counts for the same annotations were calculated using the custom Python script in Xiao et al.17, which retains only uniquely mapped reads occurring within the middle of the CDS region. For hg19 RefGene annotated genes, reads aligning after the first 15 codons and before the last 5 codons were counted. For protein-coding smORFs, reads aligning after the first and before the last exon were counted. Xtail was used to calculate the log fold changes in TF binding or thapsigargin-treated versus untreated controls. Xtail uses a binary ‘homodirectional’, ‘transcription-only’ or ‘translation-only’ category of differential translation. DESeq2 (ref.24) was also run in parallel with Xtail to calculate differential mRNA expression for hg19 RefGene annotations and smORFs. Plots summarizing the results from both analyses were generated using R.

PhyloCSF and BLAST analyses of protein-coding smORFs. Smoothed PhyloCSF scores for the 29-mammals alignment were extracted for all smORFs from the UCSC genome browser’s PhyloCSF Track Hub using the bedtools map function. The scores represent the log-odds that codons in the smORF are in the coding state. The average smoothed PhyloCSF scores are shown for each protein-coding smORF by exon (Supplementary Data 1).

All smORFs were queried for similarity against the non-redundant database using BLASTn and BLASTp under default parameters. BLAST alignments were considered significant if the BLAST score was ≥80 or ≥80% of the microprotein sequence matched ≥80% of the aligned subject sequence. This second condition allowed for the identification of short but high similarity sequence alignments, which otherwise would have a low BLAST score.

Microprotein domain predictions. Microprotein sequences were assessed for possible transmembrane helices using TMHMM v2.0 (ref.25) under default parameters. BLAST alignments were considered significant if the BLAST score was ≥80 or ≥80% of the microprotein sequence matched ≥80% of the aligned subject sequence. This second condition allowed for the identification of short but high similarity sequence alignments, which otherwise would have a low BLAST score.

Mass spectrometry data analysis. Mass spectrometry data from ProteomeXchange (PXD000394)46 were downloaded from the PRIDE archive. Tandem mass spectra were extracted from RAW files using RawConverter v1.0.0.0. Next, the
spectra were searched against a database containing human Swiss-Prot proteins, microproteins and common contaminants using ProLuCID\textsuperscript{57}. The enzyme specificity was set to none and no variable modifications were included. The false discovery rate was set to 1% for peptides. Identified spectra were then filtered and grouped into proteins using DTASelect\textsuperscript{58}. Mass spectrometry analyses were separated by different cell lines from the study. We also utilized the pFind 3 Open-pFind\textsuperscript{59} search engine to identify microprotein-derived peptides by an open search strategy, which allows for many variable modifications, using the same database and false discovery rate.

**HLA-I peptide-binding assay.** The affinities of microprotein-derived peptides for HLA-I were measured as previously described\textsuperscript{60}. Briefly, SupB15 cells (HLA-I: HLA-A3, B11, B51, B52 serotype) were collected and the cell surface HLA complex was disassembled by treating with citric acid elution buffer (pH 2.9) for 90 s. Then, cells were incubated with a high-affinity fluorescein-labeled reference peptide KVFPCC(FITC)ALINK (1 μM) and increasing concentrations of a nonlabeled microprotein-derived peptide for 20 h at 4 °C. A negative control peptide from the recently characterized microprotein NoBody\textsuperscript{13} (TPNGGSTTL, B7 serotype binder) was also tested for comparison. Fluorescence intensities were measured by flow cytometry. Binding of the microprotein-derived peptides at each concentration was calculated as percentage inhibition of reference peptide binding relative to background (without reference peptide, MF\textsubscript{bg}) and the maximal response (reference peptide only, MF\textsubscript{ref}) using the following equation:

\[
\text{Inhibition} = \left(1 - \frac{\text{MF} - \text{MF}_{\text{bg}}}{\text{MF}_{\text{ref}} - \text{MF}_{\text{bg}}}\right) \times 100
\]

The data were then plotted and fit for half-maximum inhibitory concentration (IC\textsubscript{50}) calculation using Prism\textsuperscript{5}.

**Peptide synthesis.** Peptides were purchased from Peptide 2.0. The fluorescein-labeled reference peptide KVFPCC(FITC)ALINK was synthesized by covalently coupling fluorescein to the cysteine residue with 5-(iodoacetamido)fluorescein (Marker Gene Technologies, catalog no. M0638) for use in the HLA-binding assay. All peptides were purified by high-performance liquid chromatography and confirmed by mass spectrometry.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All sequencing datasets generated in this study are available through GEO (GSE125218).

**Code availability**

A custom java script used for three-frame in silico translation of assembled transcripts is included as Supplementary Data 4.

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**Author contributions**

T.F.M. and A.S. conceived the project, designed the experiments and wrote the manuscript. T.F.M. performed cell culture and prepared RPFs and total RNA. T.F.M. and C.D. prepared Ribo-seq libraries. T.F.M. analyzed Ribo-seq and RNA-seq data, developed the smORF annotation workflow and wrote custom scripts to generate Ribo-seq plots. M.N.S. performed HLA-I experiments. T.F.M. and D.T. analyzed HLA-I proteomics data. All authors discussed the results and edited the manuscript. A.S. supervised the study.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Correspondence and requests for materials should be addressed to T.F.M. or A.S.

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Software and code

Policy information about availability of computer code

Data collection

Raw sequencing data was demultiplexed and converted into FASTQ files using CASAVA (v1.8.2). Reads were trimmed of adaptor sequences using fastx_clipper (0.10.13) and aligned with STAR (2.5.2b). Read alignments were processed using samtools (1.3).

Data analysis

Ribo-Seq data was analyzed for smORF translation using RibORF (v0.1). Translated smORFs were filtered against annotated CDS regions using bedtools (2.25). Differential expression analysis was performed using DESeq2 (1.14), and differential translation was analyzed using Xtail (1.1.5). Mass spectrometry data was searched using ProLuCID with DTASelect2 using the web interface from Integrated Proteomics Pipeline (IP2) and pfFind (3.1.3). De novo transcriptome assembly was carried out using cufflinks. Generation of ORF databases from the de novo assembled transcriptome of each cell line was done using a custom java script, GTToFASTA. Bar graphs and curves were illustrated by GraphPad Prism 7 and R (3.3).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: Human smORF annotation by Riboseq is novel and therefore the field does not have accepted guidelines for how many replicates to use. Because we found hundreds to thousands of new smORFs with each of the first 3 replicates, we collected and analyzed a minimum of 3 replicates for each cell line. Beyond that we found fewer novel smORFs to be detected. For transcriptome assembly using RNA-Seq, two biological replicates each prepared using two different RNA-Seq library methods (4 total libraries) were utilized for each cell line in order to ensure comprehensive coverage and accuracy.

Data exclusions: No data points were excluded from our analyses.

Replication: For smORF annotation experiments, the overlap was measured and reported for all experiments. For differential mRNA expression and translation experiments, known ER stress genes were checked for similar changes in expression or translation. Differential expression and translation software calculate adjusted p-values for changes. For differential expression $p_{adj} < 0.05$ was used, while $p_{adj} < 0.1$ was used for differential translation.

Randomization: Randomization was not relevant to this study. Only human cell lines were studied.

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| ☑ Palaeontology                 | ☑ MRI-based neuroimaging |
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Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | All cell lines used were purchased directly from ATCC (HeLa-S3), GE Life Sciences (HEK293T), and Sigma Aldrich (K562). |
|---------------------|-------------------------------------------------------------------------------------------------------------|
| Authentication      | The cell lines are authenticated by the supplier.                                                            |
| Mycoplasma contamination | Cell lines were not tested for mycoplasma after receiving from the supplier.                                |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used.                                                            |