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1 **Standardized annotation of translated open reading frames**

2

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86
87 **To the editor:** Ribosome profiling (Ribo-seq) has extended our understanding of the
88 translational 'vocabulary' of the human genome, discovering thousands of open reading frames
89 (ORFs) within long non-coding RNAs (lncRNAs) and presumed untranslated regions (UTRs) of
90 protein-coding genes. However, reference gene annotation projects have been circumspect in
91 their incorporation of these ORFs due to uncertainties about their experimental reproducibility
92 and physiological roles. Yet, it is clear that certain 'Ribo-seq ORFs' make stable proteins, others
93 mediate gene regulation, and many have medical implications. Ultimately, the absence of
94 standardized ORF annotation has created a circular problem: while Ribo-seq ORFs remain
95 unrecognised by reference annotation databases, this lack of recognition will thwart studies
96 examining their roles. Here, we outline a community-led effort involving Ensembl / GENCODE,
97 HGNC, UniProtKB, HUPO/HPP and PeptideAtlas to produce a standardized catalog of 7,264
98 human Ribo-seq ORFs, a path to bring protein-level evidence for Ribo-seq ORFs into reference
99 annotation databases, and a roadmap to facilitate research in the global community.
100
101 Ribo-seq[1] provides an RNA sequencing-based readout of mRNA translation by isolating
102 ribosome-bound RNA fragments of ~30 nucleotides in length. Sequencing of these fragments
103 offers genome-wide footprints of ribosome–RNA interactions, detecting translated ORFs with
104 sub-codon resolution[2–8]. Although Ribo-seq circumnavigates the experimental difficulties of
105 working with protein molecules (e.g., using mass spectrometry (MS) analytical tools) and readily
106 finds translations missed by *in silico* evolutionary methods, it does not demonstrate actual
107 protein existence, and most translations do not show signs of constraint as coding sequences
108 (CDS). A wide range of 'functional' scenarios are therefore plausible for Ribo-seq ORFs (**Table
109 1**).
110
111 Several public resources already process and/or display Ribo-seq datasets, including sORFs.org[9],
112 GWIPS-viz[10] and Trips-Viz[11], whereas OpenProt[12] and nORFs.org[13] incorporate Ribo-seq into

113 whole translatome catalogs. Meanwhile, McGillivray *et al.* have produced a catalog of upstream
114 ORFs (uORFs) with predicted biological activity[14]. Such efforts have made important
115 contributions in Ribo-seq ORF interpretation. Nonetheless, the global scientific community is
116 constrained by the absence of 'reference' gene annotation, which supports most large-scale
117 genomics projects and provides the framework for human variant interpretation (**Fig. 1a,**
118 **Supplementary Fig. 1**).
119
120 The creation of Ribo-seq annotations within existing reference gene and protein databases
121 presents specific challenges that were not faced by previous cataloging efforts[9–13]. In particular,
122 we must consider how these annotations can be integrated into the broad range of user
123 workflows that are already supported by global annotation resources. For such reasons, reference
124 annotation projects are generally conservative when it comes to the incorporation of new data
125 types. Thus, rather than attempt to describe a 'maximal' set of potential Ribo-seq translations
126 from the outset, our strategy is to build up a comprehensive resource in stages that is reciprocally
127 improved by input from the scientific community (**Fig. 1b**).
128
129 Here, as 'Phase I' of this work, we present a consolidated catalog of Ribo-seq ORFs from seven
130 publications[2–8] annotated onto GENCODE v35 (**Fig. 1c; Supplementary Tables 1–9**). A
131 detailed description of the Ribo-seq datasets, our analysis methods, and ORF characteristics is
132 available in the **Supplementary Methods**. We removed ORFs under 16 amino acids (aa) and
133 those translated from non-ATG ('near-cognate') initiation codons, and merged redundant sense
134 overlapping ORFs, resulting in a collated set of 7,264 unique ORFs (**Fig. 1c**). We classified these
135 ORFs according to their spatial relationship with existing gene annotations (**Fig. 1d**), as
136 presented in **Table 2**. We hope community usage of this catalog will help address the key
137 technical and biological questions necessary to move this work into 'Phase II', where we aim to
138 create a more comprehensive resource as outlined below.
139
140 For Phase I, we investigated repeated ORF identifications between studies, observing that 3,085
141 of 7,264 Ribo-seq ORFs were found by more than one publication (**Supplementary Fig. 2;**
142 **Supplementary Tables 2,3**). However, whereas such 'reproducibility' would demonstrate
143 consistency in Ribo-seq signal, it neither provides insights into biological function, nor indicates

144 that the 4,179 non-replicated ORFs are ‘false’. A major goal of Phase II will be to incorporate a
145 greater diversity of human cell types and tissues for improved estimates of ORF reproducibility,
146 expression patterns, and potential cell-type specificity, along with further evaluation of criteria to
147 quantify the technical confidence in Ribo-seq ORF calls.
148
149 Furthermore, Phase I excluded many translations by restricting the consensus set to ATG-
150 initiated ‘cognate’ translations of at least 16 aa in length. Although these tiny ORFs may provoke
151 skepticism in the absence of additional evidence — the smallest annotated human protein is 24
152 aa — there may be no lower size limit for a functional ORF[16]. For example, the tarsal-less (*tal*)
153 gene produces a polycistronic transcript translated into proteins as short as 11 aa in several insect
154 species[15]. Furthermore, the inclusion of ORFs initiated with near-cognate start codons can be
155 complicated by ambiguous predictions of initiation site positions[17]. Ribo-seq following treatment
156 with lactimidomycin or homoharringtonine, which inhibit translation elongation and result in
157 accumulation of sequencing reads at the putative start sites, can help to identify near-cognate
158 start sites[17,18]. Such datasets will be leveraged by our future Phase II efforts. For our current
159 annotation resource, we have separately aggregated the Ribo-seq ORFs with near-cognate start
160 codons or translations shorter than 16 codons (**Supplementary Fig. 3a–c; Supplementary**
161 **Tables 4,5**), rather than including them in the Phase I catalog.
162
163 A core aim of Phase II will be to identify which Ribo-seq ORFs participate in cell physiology
164 and how they do so. One aspect is distinguishing between cellular function mediated by a stable
165 protein versus functionality imparted at the level of translation itself. We here use ‘protein’ as an
166 umbrella term for protein, peptide and polypeptide, although we recognize that the terms
167 polypeptide, micropeptide, or microprotein are commonly used for small protein molecules
168 (**Table 2**). Because of the challenges of protein sequencing, evolutionary analysis has played a
169 major historical role in ORF annotation, which is based on the assumption that the evolution of
170 translated sequences is driven by selection at the protein level. Within our **Phase I** dataset, 75
171 Phase I replicated Ribo-seq ORFs (2.4%) present evidence of potential protein-level constraint as
172 measured by PhyloCSF[19] (**Supplementary Fig. 3d-f**), 10 of which have now been classified as
173 protein coding by GENCODE (**Supplementary Table 6**).
174

175 However, the evolutionary profile of many Phase I Ribo-seq ORFs remains hard to interpret. In
176 part, this is because distinguishing ORF selection at the protein and DNA levels can be
177 especially difficult for very small regions, noting that Ribo-seq ORFs are typically much smaller
178 than known annotated proteins (**Supplementary Fig. 3g-j**). A second drawback is that
179 evolutionary analysis cannot infer the protein-coding or regulatory potential of evolutionarily
180 'young' de novo Ribo-seq ORFs[20]. Reference annotation projects remain skeptical on the
181 existence of proteins that are not deeply conserved, despite the fact that some young proteins
182 clearly do participate in cellular physiology[20,21]. Furthermore, there is a substantial knowledge
183 gap on the mode and tempo of regulatory ORF evolution. Here, genetic variation within human
184 populations may provide insights. For example, Whiffin *et al*[22] recently used the gnomAD
185 human variation dataset to identify 3,191 genes where uORF-perturbing variants are likely to be
186 deleterious, thereby inferring the physiological importance of these translations. Meanwhile
187 Neville *et al.*[23] used the same dataset to find aggregate evidence of selective pressure against
188 deleterious variants in their nORFs.org catalog[13], especially pronounced for STOP-gain variants
189 in uORFs. In prostate cancer, a recent analysis of 5′ UTR variants found regulatory roles for
190 several uORFs[23].
191
192 While Ribo-seq ORFs may have regulatory roles irrespective of an encoded protein, the first step
193 in confirming a protein-level physiological role for a Ribo-seq ORF is to demonstrate the
194 existence of the protein in the cell. Mass spectrometry (MS) is a widely-accepted approach to
195 catalogue the proteome, and its utility will be an important area of investigation for Phase II. At
196 present, 609 of 7,264 Ribo-seq ORFs were reported to have support by published MS datasets
197 (**Supplementary Table 10**). However, different groups use distinct methodologies and
198 parameters for MS, and for Phase I these findings are simply reported in **Supplementary Tables**
199 **2 and 3** without further investigation. Reference annotation projects have historically favoured
200 high stringency MS approaches, and the Human Proteome Organization (HUPO) / Human
201 Proteome Project (HPP) — which aims to produce a full annotation of the human proteome —
202 has published guidelines to standardize the nature of MS evidence required to annotate a human
203 protein[24]. As one facet of our development of an MS workflow, these Ribo-seq ORFs have been
204 added to the PeptideAtlas analytical pipeline, as used by HUPO. For Phase II, our projects will
205 jointly examine the question of how best to use MS data to define which Ribo-seq ORFs produce

206 proteins. For reference annotation, we see two aspects to this: first, how to set standards for
207 accepting and reporting potential MS support for a prospective Ribo-seq ORF protein; and
208 second, how to define the point at which the body of evidence supports protein-coding
209 annotation.
210
211 These aspects are illustrated by a preliminary analysis, which took advantage of the fact that 333
212 of our Ribo-seq ORFs are present in sequences previously queried by the PeptideAtlas workflow
213 (**Supplementary Methods**). We find single-mapping peptide-spectrum matches (PSMs) for 13
214 Ribo-seq ORFs (**Supplementary Table 11**); all except one is supported by a single PSM,
215 whereas most of the peptides identified are not fully tryptic (two examples are presented in
216 **Supplementary Fig. 4**). The majority of observed PSMs derive from human leukocyte antigen
217 (HLA) peptidome datasets, which is consistent with prior proteomic analyses demonstrating
218 enrichment for peptides mapping to Ribo-seq ORFs in immunopeptidome data[25–27]. We
219 emphasise that this preliminary analysis was not a full remapping of MS data and contained a
220 fraction of the Ribo-seq ORFs; a larger, focused effort will be forthcoming.
221
222 There are multiple aspects as to why Ribo-seq ORFs and certain classes of canonical proteins are
223 infrequently detected in MS data, which are summarized elsewhere[28]. One consideration for
224 HUPO is that an MS-based ‘canonical’ protein assignment requires multiple PSMs, ideally based
225 on non-overlapping tryptic peptides. Although we recognise the value of these guidelines, very
226 small proteins may be ‘less discoverable’ by MS, especially due to a paucity of identifiable
227 tryptic fragments[28]. Notably, nearly 1,500 protein-coding genes annotated by GENCODE,
228 UniProt and HGNC do not presently have MS support recognised by HUPO[24]. Moving forward,
229 we are committed to examining all potential protein-coding Ribo-seq ORF cases with full
230 manual gene annotation processes, and this workflow will be expanded to include manual
231 analysis of the peptide spectra by PeptideAtlas.
232
233 Although the value of MS in identifying translated proteins is indisputable, we believe a broader
234 ‘gold standard’ for evidence should employ additional methodologies, such as epitope tagging
235 combined with western blot imaging or endogenous antibody work; HUPO already incorporates
236 such data in collaboration with the Human Protein Atlas[24]. Consideration will also be given to

237 emerging proteomics technologies, such as targeted proteomics workflows and
238 immunopeptidomics, whereas progress is being made in medium-throughput functional
239 screening assays. For example, recent large-scale studies have translated hundreds of Ribo-seq
240 ORFs in mammalian cells through exogenous expression, finding that nearly 50% may stably
241 produce proteins, despite little evidence of evolutionary constraint[2,6,27].
242
243 In addition to their evaluation as proteins or regulatory units, the reference annotation of Ribo-
244 seq ORFs necessitates the creation of integrated workflows to interpret overlapping variants, and
245 notwithstanding great community interest in this field, standardised approaches are not yet
246 available. We emphasise that variant interpretation pipelines designed to classify CDS mutations
247 may be unsuitable for Ribo-seq ORFs (**Table 1**), and that a minority of overlapping variants fall
248 within sequences displaying amino acid-level constraint. Neville *et al.*[13] found that their
249 nORFs.org catalog contains 48 Human Gene Mutation Database or ClinVar variants already
250 considered pathogenic or likely pathogenic, despite the fact that they do not disrupt annotated
251 CDSs. Although these variants may affect non-canonical ORFs, it will be important to define
252 their mechanisms of action with experimental studies, as alternative explanations for
253 pathogenicity are supported in certain cases, such as the creation of cryptic splice sites. After
254 excluding variants in Ribo-seq ORFs that overlap annotated CDSs, a total of 1,142 single
255 nucleotide variants present in the ClinVar database[29] were located within our aggregated set of
256 Phase I Ribo-Seq ORFs (**Supplementary Methods**). Fewer than 2% of these variants have been
257 classified as pathogenic or likely pathogenic, but this is likely to be an underestimate because the
258 absence of pathogenesis is commonly inferred due to the absence of overlap with known coding
259 features, and because ClinVar variant coverage is heavily skewed towards annotated CDSs.
260
261 Furthermore, there is major interest in the usage of Ribo-seq for the study of human disease. In
262 particular, it is being widely used to explore the dynamics of translation in cancer cells with
263 aberrant proteins as diagnostic markers or targets for immunotherapy[25,26,30]. At present, reference
264 annotation projects do not attempt to distinguish aberrant translation from those events that
265 contribute to 'normal' physiology. It will be important to deduce the fraction of Ribo-seq ORFs
266 that encode proteins that exist in normal cellular conditions. Conversely, we envisage the value

267 of classifying potentially aberrant translations within Phase II through a distinct annotation
268 framework.
269
270 Our intention is for the Ribo-seq Phase I catalog to be seen as a pragmatic interim solution to a
271 long-term problem. We believe that reference annotation databases can advance both scientific
272 and clinical research through the propagation and standardization of Ribo-seq ORF datasets,
273 even — and perhaps especially — while the phenotypic impact of these features remains
274 uncertain. As biological knowledge improves, this will support the development of more
275 accurate annotations and variant interpretations, with the potential to yield substantial insights
276 across all aspects of human biology. In this spirit, we hope the results of Phase I of this project
277 will be useful and beneficial to the community and invite interested labs to join our future Phase
278 II efforts.
279

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Author contributions

JMM, JR-O, JRP, SvH conceptualized the work and supervised the international collaboration. JR-O, JMG, MM, MJM, FRC, EB, EWD, RLM, JMM performed data curation. All authors contributed to standardization of the data analysis approach. All authors contributed to discussions on Phase I and II of this effort and continue to provide scientific oversight. AF, PF, MJM, M, YTY, JRP, TFM, MMA, JC, JSW, AAB, AR, PVB, NH, XR, MAB, NTI, EB, EWD, RLM provided funding. JMM, JR-O, JRP, SvH wrote the original manuscript draft. All authors reviewed the manuscript and provided edits. All authors approved the final manuscript.

Competing interests

PVB is a co-founder of RiboMaps Ltd that provides Ribo-seq analysis as a commercial service and this includes identification of translated ORFs. ARC is a member of the scientific advisory

341 board for Flagship Labs 69, Inc. PF is a member of the scientific advisory boards of Fabric
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410

411 **Table 1: Approaches to interpret Ribo-seq ORFs**

412

Possible cellular interpretation of Ribo-seq ORF translation	**Comments**
A Ribo-seq ORF encodes a 'missing' conserved protein	Ribo-seq ORFs may be recognised as canonical – in accordance with existing protein annotations – on the basis that the sequence of the protein they encode shows clear evidence of being maintained by evolutionary selection over a significant period of evolutionary time.
A Ribo-seq ORF encodes a taxonomically restricted protein.	Ribo-seq ORFs may encode proteins whose sequence and molecular activities are specific to one species or lineage. Evidence for selection or conservation across distant species or lineages is lacking for these ORFs, either because the protein sequence has diverged beyond recognition from its orthologues, or because the protein evolved recently from previously noncoding material and homologues do not exist in other species or lineages.
A Ribo-seq ORF regulates protein or RNA abundance.	Ribosome engagement of regulatory ORFs does not result in a protein product under selection but regulates the abundance of a canonical protein or RNA. This paradigm is well established for uORFs and uoORFs, as noted in **Table 2,** though it is applicable to other transcript scenarios. Regulatory ORFs

	may compete for ribosomes with their downstream canonical ORFs or produce nascent peptides that stall ribosomes, leading to the controlled 'dampening' of protein expression. Alternative modes of action, such as the induction of RNA decay pathways, the processing of small RNA precursors or the adjustment of RNA stability, have also been inferred.
A Ribo-seq ORF is the result of random translation.	The translation of some Ribo-seq ORFs may simply be 'noise'. Since translation has a high bioenergetic cost, a protein that results from random translation is likely to be translated at lower levels than a canonical CDS and evolve neutrally; it may also be unstable in comparison, and be potentially rapidly degraded. Nonetheless, it is theoretically possible that certain proteins do exist as stable 'junk' proteins, or that random translation events affect the expression of the canonical protein. The detection of random Ribo-seq ORFs is less likely to be reproducible.
A Ribo-seq ORF encodes a disease-specific protein.	This protein would not be produced under normal physiological homeostasis but could be of major interest for diagnostics and therapeutics**.** Insights are especially emerging in cancer biology, where transcription and translation are known to be dysregulated. This leads to the production of 'aberrant', possibly

	rapidly-degraded proteins that are commonly antigenic and presented on the cell surface by the HLA system, offering the prospect of neoantigens. In addition, antigens resulting from disease-specific dysregulated ribosome activity - sometimes called defective ribosomal products (DRiPs) - have also been explored.

413 Note: a given ORF may encompass several of these possibilities, e.g., a translated ORF that is
414 both regulatory and implicated in disease neoantigen production.
415

416 **Table 2. Terminology and categories of Ribo-seq ORFs**

417

Term	**Definition**	**Biological role**
Ribo-seq ORF	Translated sequences identified by the Ribo-seq assay that have not already been annotated by reference annotation projects Also known as: non-canonical ORFs, alternative ORFs (altORFs), novel ORFs (nORFs). If <100 amino acids in size: small ORFs (smORFs), short ORFs (sORFs). Putative encoded proteins in smORFs/sORFs are also known as: microproteins, micropeptides, short ORF-encoded polypeptides (SEPs).	See below
Upstream ORFs (uORFs)	Translated sequences located within the exons of the 5' untranslated region (5' UTR) of annotated protein-coding genes.	Regulation of the translational efficiency of the downstream canonical protein. Cellular stress-related translation. May produce independently-functional proteins.
Upstream overlapping ORFs (uoORFs)	Translated sequences beginning in the 5' UTR of an	Similar to uORFs. Regulation translation of their

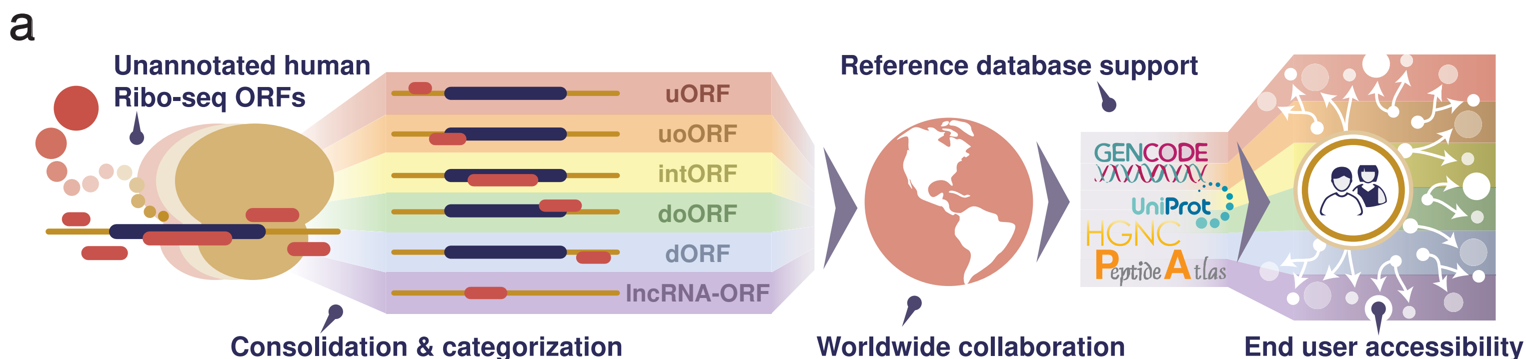
	annotated protein-coding gene and partially overlapping its coding sequence in a different reading frame.	overlapping CDS, but potentially stronger regulatory potential compared to uORFs. May produce independently-functional proteins.
Downstream ORFs (dORFs)	Translated sequences located within the 3’ UTR of annotated protein-coding genes	Less commonly detected and generally poorly understood. May act as *cis* translational regulators.
Downstream overlapping ORFs (doORFs)	Translated sequences beginning in the genomic coordinates of an annotated CDS but continuing beyond the annotated CDS and terminating in the 3’ UTR of the annotated protein-coding gene.	Similar to dORFs
Internal out-of-frame ORFs (intORFs)	Translated sequences located on the mRNA of an annotated protein-coding gene and completely encompassed within the canonical CDS, but translated via a different reading frame. Also known as: altCDSs, nested ORFs, dual-coding regions.	May regulate translation similar to uORFs in some cases. Detection of intORFs with Ribo-seq is possible but difficult due to the convolution of triplet periodicity signals from two reading frames; it largely depends on the length and translation level of the intORF relative to the overlapping canonical CDS.

Long non-coding RNA ORFs (lncRNA-ORF)	Translated sequences located within transcripts currently annotated as long non-coding RNAs (lncRNAs), including long intervening/intergenic noncoding RNAs (lincRNAs), long non-coding RNAs that host small RNA species (encompassing microRNAs, snoRNAs, etc), antisense RNAs, and others	May produce independently-functional proteins. Typically lack strong sequence conservation.

418

419 **Figure 1.** Characterization of a consensus set of Ribo-seq ORFs for annotation by GENCODE.
420 **(a)** A schematic of the main steps and goals for this consortium effort. **(b)** A map showing the
421 participating institutions included in this effort. **(c)** A schematic overview of employed filtering
422 steps used to create the consensus set of ribosome profiling (Ribo-seq) ORFs. **(d)** A
423 diagrammatic representation of all Ribo-seq ORFs according to ORF type (see **Table 2** for more
424 information).
425

a



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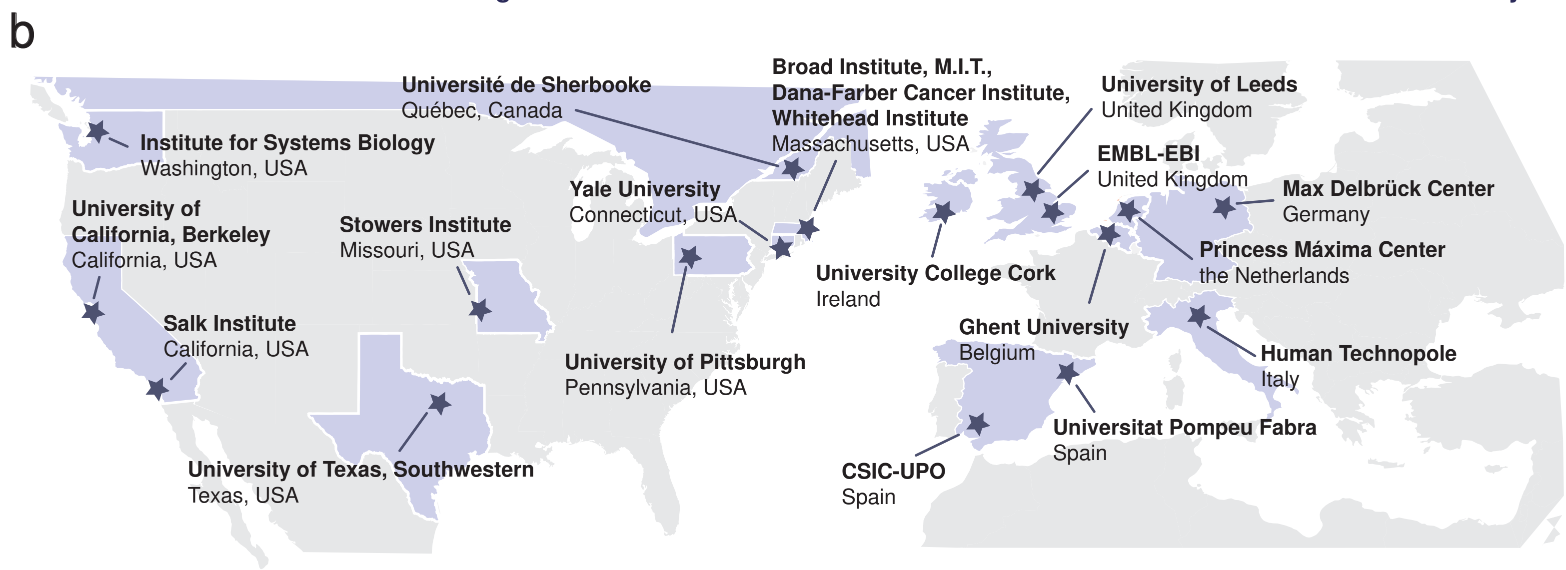

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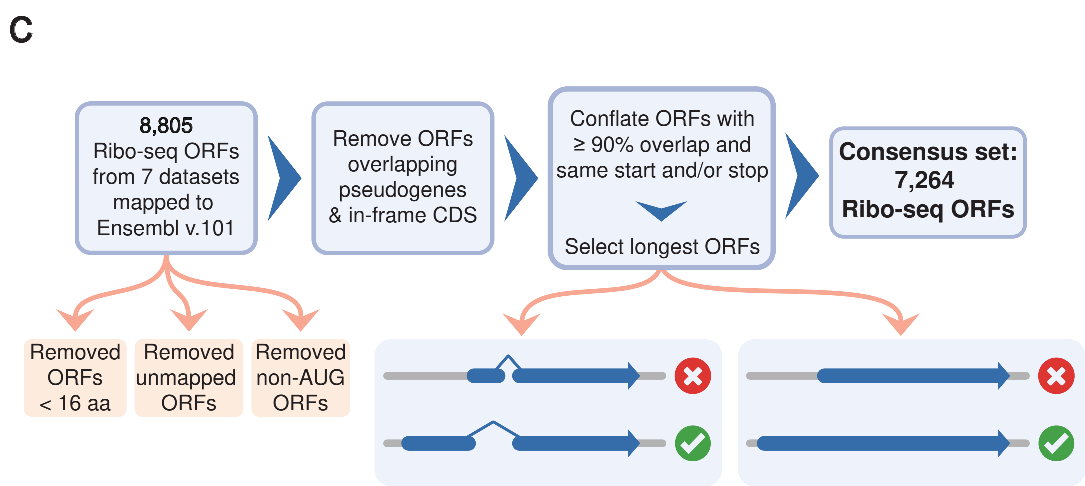

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