Evolutionary remodeling of non-canonical ORF translation in mammals

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Abstract

Non-canonical open reading frames (ncORFs) are pervasive within transcripts annotated as "non-coding" or "untranslated regions" of mRNAs, yet their landscape under normal physiological conditions remains to be fully resolved, particularly outside humans. Here we applied a stringent and standardized pipeline to hundreds of high-quality ribosome profiling libraries from normal mammalian tissues and cell types, identifying 11,623 human and 16,485 mouse ncORFs. Evolutionary analyses revealed that thousands of ncORFs are subject to coding constraint and exhibit lineage-specific conservation, underscoring their functional potential. Ancient ncORFs are preferentially highly translated, broadly expressed, and enriched for lineage-specific conservation. Co-expression patterns further indicate that many ncORFs, especially ancient ones, are cotranslated with canonical coding sequences, consistent with functions mediated through protein–protein interactions. Together, these findings establish a comprehensive atlas of mammalian ncORFs and provide fundamental insights into their evolutionary dynamics and functional integration within the proteome.

Keywords: non-canonical open reading frame; annotation; translation; evolution; mammals.

Introduction

The systematic identification of functional genomic elements represents a foundational objective in the field of genomics. Among these elements, non-canonical open reading frames (ncORFs)—which are open reading frames (ORFs) located within long non-coding RNAs (lncRNAs) or untranslated regions (UTRs) of mRNAs—have attracted considerable attention in recent years[1-5]. While certain ncORF subtypes, such as upstream ORFs (uORFs) located in 5' UTRs and downstream ORFs (dORFs) in 3' UTRs, have been extensively characterized for their ability to regulate mRNA translation and stability in *cis*, a growing body of evidence demonstrates that ncORFs can also give rise to functional proteins that engage in diverse cellular pathways[1,2,6,7]. As functional ncORFs continue to be discovered across multiple eukaryotes[8-17], systematic elucidation of their composition and function promises to uncover an additional layer of regulatory complexity and to refine our conception of the so-called "non-coding" genome.

Although ncORFs may play important biological roles, the number of translated ncORFs in the human genome remains uncertain. Detection by mass spectrometry (MS)-based proteomics is challenging, likely due to their short lengths and low abundances[18]. Ribosome profiling (Ribo-Seq) offers greater sensitivity for identifying translation events[18,19], but downstream computational pipelines often produce highly divergent ncORF sets [20]. Consequently, published estimates of ncORF abundance span several orders of magnitude—ranging from thousands to millions [15-22]. To establish a standardized reference, the GENCODE consortium integrated data from seven distinct studies to produce a catalog of 7,264 human ncORFs (hereafter GENCODE ncORFs)[2]; however, the heterogeneity of sample sources and prediction algorithms likely introduced systematic bias. This catalog is also restricted to AUG-initiating ORFs, thereby overlooking those with near-cognate start codons. A later effort generated a high-resolution human translatome map and identified 7,767 ncORFs with a unified pipeline[22]. Nevertheless, this conservative annotation disproportionately under-represents coding sequence (CDS)-overlapping ncORFs. Indeed, later estimation suggests that between 10,500 to 22,500 ncORFs should exist in the human genome[18]. Furthermore, many ncORFs were reported from studies of cancer cells[1,13,23,24], where extensive transcriptome reprogramming alters the translational landscape[25,26]. While paired healthy tissues were also analyzed in these studies, the landscape of ncORFs translated under normal physiological conditions is not yet fully resolved.

While a limited subset of ncORFs has been shown to encode signaling molecules, cofactors, membrane proteins, or subunits of large protein complexes[1,27,28], functions of most ncORF-encoded proteins (ncEPs) are largely uncharacterized. Advances in high-throughput screen technologies have enabled the systematic interrogation of functional ncEPs[23,28-32]. Nevertheless, there is minimal concordance among hits from different studies[3]. Evolutionary analyses can offer insights into potential functionality of these translation products and mechanisms of origination[33-35], yet how ncORF translation has been remodeled to integrate into proteome since their evolutionary origins is still poorly understood. Consequently, the extent to which translated ncORFs encode functional proteins and the

evolutionary dynamics of their integration into the existing proteome are largely unresolved. Moreover, because most ncORF studies have focused on humans, it is unclear whether ncORFs in other species share similar sequence features and expression patterns.

To address these gaps, we generated a standardized annotation of translated ncORFs by uniformly reprocessing ~400 high-quality ribosome profiling libraries from normal mammalian tissues and cell types. This stringent approach yielded 11,623 human and 16,485 mouse ncORFs, consistently recovered across diverse samples and prediction strategies. Comparative and functional genomic analyses revealed that thousands of these ncORFs are under evolutionary constraint and exhibit distinct translation profiles. The resulting dataset provides a robust, comprehensive resource that will facilitate future investigations into the biological roles of ncORFs across cellular and organismal contexts.

Results

Annotation of ncORFs translated in normal mammalian cells

We developed a standardized pipeline to systematically annotate ncORFs translated in normal human and mouse cells (Fig. 1A). We first curated publicly available Ribo-Seq libraries, excluding those from pathological or genetically modified samples. Since both library size and the proportion of in-frame ribosome-protected fragments (RPFs) affect the sensitivity and accuracy of ncORF prediction[20], we filtered out libraries containing fewer than 5 million RPFs or with in-frame rates below 60%. After this quality control step, we retained 174 human and 209 mouse high-quality Ribo-Seq libraries, spanning a range of tissues and cell types (Fig. S1A; Table S1). We predicted translated ORFs that begin with NUG (where N stands for any nucleotide) start codons and encode at least five amino acids using three tools—PRICE[36], RiboCode[37] and Ribo-TISH[38]—which we previously benchmarked for optimal performance[20]. After excluding annotated CDSs and their extension or truncation variants arising from alternative initiation sites, we obtained a preliminary list of ncORFs in each library.

To generate reliable ncORF annotations, we applied stringent filtering and integration criteria. We removed potential false positives caused by non-ribosomal contamination or translational noise based on metrics including Fragment Length Organization Similarity Score (FLOSS)[39], Ribosomal Release Score (RRS)[40,41] and the number of RPFs within an ORF by P-site. We then aggregated the remaining ORFs to identify those consistently detected across multiple methods and libraries in human and mouse datasets, respectively (Fig. 1A). In many cases, two ORFs predicted from different libraries or methods may share the same start or stop codon due to the alternative translation initiation or splicing, resulting in groups of overlapping ncORFs. For example, 11.1% (805) of GENCODE ncORFs overlap with at least one other ORF, forming 329 overlapping groups (Table S2). This overlap complicates the identification of reproducible ORFs, as ORFs within a group may represent distinct proteoforms of a protein. To address this issue, we collapsed each group of ncORFs sharing the same start or stop codon into a single cluster, and retained only those clusters supported by at least two independent methods and observed in more than one library. For each retained cluster, we selected a representative ORF by

prioritizing transcript annotation[42,43], start codon type, and ORF length to facilitate downstream analyses (Fig. 1A).

This approach yielded 12,469 translated ncORFs in humans and 16,960 in mice. However, manual inspection revealed a subset of spurious ncORFs that are not truncation or extension variants of annotated CDSs, but instead partially overlap with annotated CDSs from alternative transcript isoforms in frame (Figs. 1B & C). For instance, in gene *AGTPBP1*, the CDS of transcript *ENST00000357081* begins downstream of *ENST00000628899* CDS due to the inclusion of an alternative exon. As a result, the 5' UTR of *ENST00000357081* appears to contain a translated uORF (Fig. 1B), which overlaps in-frame with the CDS of *ENST00000628899* and may be mistakenly identified as a translated ncORF owing to active translation of the latter. A similar case is seen with the lncRNA ORF (lncORF) in *ENST00000462211*, which overlaps in-frame with the CDS of *ENST00000328767* from the *TMTC4* gene and may thus be erroneously identified as a translated ncORF (Fig. 1C). To avoid such artifacts, we implemented an additional filtering step to exclude these overlapping cases and left with 11,623 and 16,485 high-confidence ncORFs in humans and mice, respectively (Table S3). Notably, while more than half ncORFs were detected in two to five libraries, 22.3–26.3% were identified in more than 10 libraries, indicating a substantial subset with robust and recurrent translation signals suitable for future experimental validation (Fig. S1B).

Annotated ncORFs are supported by independent evidence

To assess whether the annotated ncORFs are supported by independent evidence, we compared them with a previously compiled catalog of ncORFs identified using mass spectrometry (MS)-based proteomic data[20]. For the 3,494 MS-supported ncORFs in humans and 873 in mice, 44.5% and 67.6%, respectively, were also predicted as translated ncORFs in this study (Fig. S2). We further evaluated our annotation against the GENCODE ncORFs[2], a widely used community resource. Of the 7,264 GENCODE ncORFs, 6,974 are located in fully annotated transcripts that were included in our analysis. Among them, 2,997 were reported by at least two independent studies (hereafter "phase 1"), while the remaining were only reported in a single study ("single")[2]. We found that 75.6% of phase 1 ncORFs were independently recovered in our annotation, significantly more than the single-study ncORFs (Fig. 1D; $P = 6.4\times10^{-307}$, Fisher's exact test).

A recent study conducted a comprehensive pan-proteome analysis to provide high-confidence peptide evidence for GENCODE ncORFs[44]. In this study, ncORFs were categorized into four tiers: tier 1 and 2 ncORFs had support from both MS and Ribo-Seq data; tier 3 had MS evidence only; and tier 4 had Ribo-Seq evidence only[45]. Notably, our annotation was significantly enriched for tier 1 or 2 ncORFs ($P = 2.0\times10^{-64}$, Fisher's exact test), with 446 and 661 of them detected, respectively (Fig. 1E). Combining MS-supported ncORFs from our previous compilation and those from GENCODE ncORFs, 2,088 (18.0%) human ncORFs identified in this study were supported by MS evidence. Despite this

overlap, the majority (65.2%) of human ncORFs in our annotation are not included in either of the two catalogs. This highlights both the complexity of the non-canonical translatome and the critical role of tissue diversity in comprehensive ncORF annotation.

To further validate our findings, we manually curated ncORFs with known functions from the literature. We identified 17 ncORFs in our annotation that have been experimentally confirmed to be translated—either through reporter assays or antibody detection—and shown to produce functional proteins (Fig. 1F; Table S4). Notably, 16 of these validated ncORFs are from humans, and only 12 are included in the GENCODE catalog. This analysis further underscores the accuracy and functional relevance of our annotations. Together, these results indicate that our annotation pipeline generates a robust and reliable catalog of translated ncORFs in mammals.

ncORFs exhibit signatures of translation and encode putative proteins lacking known domains

We classified ORFs into categories based on community recommendations[2] and found that the distribution of ORF types closely aligns with previous estimates[18]. In both human and mouse, uORFs, upstream overlapping ORFs (uoORFs), and lncORFs are more prevalent than other types, whereas internal ORFs (iORFs), downstream overlapping ORFs (doORFs), and dORFs are relatively rare (Fig. 2A). Although most ncORFs are short, encoding fewer than 100 amino acids (Fig. 2B), their sequences show clear signatures of translation similar to CDSs. These include preferential usage of AUG start codons (Fig. S3A), enrichment toward 5' transcript ends (Fig. S3B), and reduced RNA secondary structure[46] (Fig. S3C) and optimized Kozak contexts[47,48] around start codons, especially in non-AUG ncORFs (Figs. S3D & S4). Despite these similarities, ncORFs differ markedly from CDSs in both codon and amino acid usage (Figs. S5A-B). This divergence is further supported by their lower tRNA adaptation index values and higher effective number of codons, indicating less optimized codon usage (Figs. S5C-D). Together, these findings show that ncORFs share core sequence and structural features with canonical CDSs, yet maintain distinct codon and amino acid usage profiles.

Although ncORFs can encode putative proteins, the functional significance of ncEPs remains poorly understood. As an initial step toward functional inference, we systematically searched for annotated protein domains within ncEP sequences, reasoning that the presence of conserved domains would suggest potential biochemical activity. Overall, only 1.29% (150) of human and 0.78% (129) of mouse ncEPs contained at least one known domain (Fig. 2C; Table S5), presumably due to their short lengths relative to canonical proteins. Consistently, domain-containing ncORFs are significantly longer than those without annotated domains (Fig. S6) and are predominantly classified as lncORFs (Fig. 2D). Unlike canonical proteins, which have relatively low intrinsically disordered region (IDR) content (median ± standard error: 14.6±0.28% in humans and 12.7±0.26% in mice), ncEPs exhibit significantly higher IDR proportions (Fig. 2E), with medians of 62.5±1.42% and 60.0±1.27%, respectively. These results indicate that most ncEPs lack structured domains and are instead dominated by IDRs.

171 Among the eight domains observed in at least five ncORFs, three are DNA-binding domains:
172 zinc finger, Krüppel-associated box, and helix-turn-helix (Fig. 2F). Because transposable elements (TEs)
173 commonly encode DNA-binding proteins required for transposition[49], we tested whether domain-
174 containing ncORFs preferentially overlap TE-derived sequences. Indeed, domain-containing ncORFs
175 were significantly enriched for TE overlap in both humans and mice (P = 5.8×10^{-4} and 3.4×10^{-17},
176 respectively; Fisher's exact tests; Fig. 2G). Although enrichment alone does not establish origin, it
177 supports a model in which a subset of longer ncORFs may have acquired modular domains through TE-
178 derived sequence contributions.
179
180 **Putative ncEPs are selectively constrained**
181 Given the scarcity of known domains in putative ncEPs, a key question is whether they are functional
182 in cells. If at least some of these ncEPs are not merely translational noise or byproducts of translation-
183 dependent functions, we would expect them to exhibit evolutionary signatures of selective constraints
184 similar to canonical CDSs. To test this, we leveraged the recent high-resolution genomic constraint
185 maps derived from both intra- and inter-species comparisons in humans [50,51]. First, we examined the
186 genomic non-coding constraint of haploinsufficient variation (Gnocchi), which quantifies selective
187 pressure by comparing observed versus expected variants across human genomes. A Gnocchi score
188 above zero indicates purifying selection[50]. Although ncORFs exhibit lower Gnocchi scores than CDSs,
189 their score remain significantly above zero and exceed those of lncRNAs (Fig. S7), suggesting that
190 ncORF sequences are under evolutionary constraint. A hallmark of functional coding regions is codon
191 position-specific constraints, but Gnocchi scores lack the resolution needed to assess such fine-scale
192 features. Therefore, we next analyzed the base-resolution conservation metric PhyloP[52] calculated from
193 multiple alignments of primate or mammalian genomes[51]. As expected, PhyloP scores in CDSs are
194 substantially higher than those in flanking UTRs and display strong three-nucleotide periodicity, with
195 the third codon position being less conserved than first and second (Fig. 3A). Although ncORFs
196 (excluding those overlapping annotated CDSs) do not show higher overall PhyloP than flanking regions,
197 they also exhibit significant three-nucleotide periodicity in both mammals (Fig. 3A) and primates (Fig.
198 S8), suggesting that at least a subset of ncORFs are subject to similar evolutionary constraints as CDSs.
199 Motivated by the above results, we next sought to identify individual ncORFs under selective
200 pressure. To this end, we determined their modes of origination[33,34] and evolutionary history through
201 comparative genomic analyses. Our analysis revealed that most ncORFs are evolutionarily young, with
202 63.0% (7,319) and 82.8% (13,508) ncORFs in humans and mice originating within the mammalian
203 lineage, respectively (Figs. 3B & S9). In line with previous studies[33,34], we estimate that at least 51.7%
204 of human and 50.8% of mouse ncORFs arise *de novo* (Table S6). We also observed that the number of
205 ncORFs originating at each ancestral node, when normalized by branch length, decreases with
206 increasing node-to-tip distance (Fig. 3C), reflecting dynamic turnover and reduced long-term retention
207 of ncORFs. To assess whether individual ncORFs are constrained for coding, we analyzed codon

208 substitution patterns within ncORFs since origination[33] with PhyloCSF[53]. PhyloCSF estimates the log-
209 likelihood ratio of a given alignment under coding versus non-coding models; positive values indicate
210 stronger similarity to conserved coding sequences. We found 13.8% (1,608) of human and 32.0% (5,281)
211 of mouse ncORFs have positive PhyloCSF scores (Fig. 3D), and 10.9% (1,264) of human and 22.6%
212 (3,721) of mouse ncORFs even have PhyloCSF exceeding 10 (Table S6), providing strong evidence
213 that these sequences may encode functional ncEPs.
214 Evolutionary dynamics can also provide insights into the function of ncORFs, as functionally
215 constrained ncORFs are less likely to be lost over time. To quantify the conservation of each ncORF
216 since its origination, we developed a local branch length score (BLS) (Fig. 4A), adapting the "global"
217 BLS metric previously used to assess uORF conservation across full phylogenies[48,54]. Unlike global
218 BLS, local BLS is designed to detect lineage-specific constraint, making it better suited for identifying
219 functional ncORFs that may be restricted to certain evolutionary branches. Using a local BLS threshold
220 of 0.9, we identified 16.3% (1,889) of human and 24.8% (4,087) of mouse ncORFs as exhibiting
221 lineage-specific conservation (Figs. 4B & S9). Notably, these ncORFs are significantly more likely to
222 show positive PhyloCSF scores (Fig. 4C), reflecting the effectiveness of local BLS. In total, we
223 identified 681 human and 1,622 mouse ncORFs that are both lineage-specifically conserved and show
224 evidence of coding potential, making them strong candidates for future functional validation. Together,
225 these findings suggest that a subset of ncORFs may encode functional proteins rather than translational
226 byproducts.
227
228 **Expression of ncORFs is shaped by their evolutionary dynamics**
229 Gene expression is a tightly regulated process, and non-functional products are unlikely to be robustly
230 expressed. To assess the expression of ncORFs, we examined their translation across diverse tissues
231 and cell lines (Table S7). On average, 71.9% of human and 48.5% of mouse ncORFs are translated with
232 reads per kilobase per million mapped reads (RPKM) ≥ 1, which is comparable to or slightly exceeds
233 the proportions observed for CDSs (Fig. S10). To investigate the influence of ncORF evolution on their
234 expression, we analyzed mean translation levels of ncORFs across different samples. We found that
235 ncORFs with higher local BLS tend to be more highly translated in both species (Fig. S11A), whereas
236 coding potential showed only a minor influence (Fig. S11B). To disentangle the effects of evolutionary
237 constraint and gene age, we stratified ncORFs by origination nodes and examined their translation levels
238 in relation to local BLS and PhyloCSF. Although older ncORFs are generally more highly translated,
239 those with higher local BLS scores consistently show elevated translation levels compared to their age-
240 matched counterparts (Figs. 5A & S12A). This suggests that lineage-specific conservation is associated
241 with enhanced and possibly optimized translation, potentially reflecting acquired functional roles.
242 Conversely, the relationship between coding potential and translation is more nuanced: younger
243 ncORFs with positive PhyloCSF scores tend to be more highly translated, while the opposite trend is
244 observed in older ncORFs (Figs. 5B & S12B). These patterns point to complex interplays between

245 evolutionary constraint, coding potential, and translational output, likely reflecting divergent selective
246 pressures across different ncORF age groups.
247 While the above analyses revealed the overall trends in ncORF translation, they overlooked the
248 heterogeneity across tissues and cell types. We found that ncORF translation exhibits strong tissue
249 specificity (Fig. S13), which may stem from either transcriptional bias in ncORF-containing genes or
250 post-transcriptional regulation specific to ncORFs. To disentangle these factors, we examined RNA
251 levels of ncORF-containing genes across different tissues[55,56]. Although transcriptional enrichment in
252 testis was evident in both humans and mice (Fig. S14), testis-biased translation was observed only in
253 mice (Fig. S13). Moreover, ncORF translation displayed significantly higher tissue specificity than the
254 transcription of their host genes in both species (Fig. 5C), indicating a substantial post-transcriptional
255 component to their regulation. We next assessed how evolutionary characteristics shape tissue
256 specificity. Older ncORFs tend to have lower tissue specificity than younger ones, suggesting they are
257 more broadly translated (Figs. 5D & S12C). Intriguingly, coding potential exhibits opposite effects
258 depending on evolutionary age: younger ncORFs with higher coding potential are more broadly
259 expressed, whereas older ones show increased tissue specificity (Figs. 5E & S12D). These patterns may
260 help to explain the lower average translation levels observed for older ncORFs across tissues. A similar
261 trend was also evident for ncORFs with high local constraint scores in mice (Fig. S12C). Collectively,
262 these results support a model in which ncORFs initially evolve widespread expression to establish
263 interactions and avoid loss[57], but may later become functionally specialized, resulting in more restricted
264 expression.
265
266 **Extensive co-translation between ncORFs and canonical CDSs**
267 Given our earlier observation that many ncEPs lack modular domains and may act via interactions with
268 canonical proteins, we next explored the landscape of co-translation between ncORFs and CDSs across
269 different tissues or cell types as we expect that interacting proteins tend to be translated simultaneously
270 spatiotemporally. Using weighted gene co-expression network analysis (WGCNA)[58], we identified
271 61,513 human and 55,449 mouse ncORF-CDS co-translated pairs. As expected, ncORFs in mRNAs
272 tend to co-translate with the main CDS of the same gene, even after excluding CDS-overlapping ones
273 (Fig. S15), consistent with previous experimental findings[28]. Among non-overlapping co-translating
274 ncORFs, only 10 in humans were dORFs; all others in both species were uORFs. Of note, co-translation
275 does not contradict the canonical model in which uORFs repress downstream CDS translation. Co-
276 translation reflects concurrent ribosome occupancy driven by overall initiation rates at the 5' end,
277 whereas repression concerns the fraction of initiating ribosomes that reach the CDS. Thus, absolute
278 translation of both ORFs may increase without altering the relative inhibitory effect of the uORF.
279 ncORF-CDS co-translation forms a highly modular bipartite network, in which one CDS may
280 be linked to multiple ncORFs and vice versa (Fig. 6A-B). Network analysis revealed that hub CDS
281 nodes—those with the most connections—in the largest modules are significantly enriched for

282 functional gene categories (Fig. 6C-D). For instance, the largest module in humans is associated with
283 chromatin-related proteins, while the second largest module in humans and the largest one in mice are
284 linked to extracellular matrix organization. Notably, ancient ncORFs (those originating before the last
285 common ancestor of mammals) are significantly more likely to co-translate with CDSs than younger
286 ones (Fig. 6E), potentially reflecting a greater likelihood of acquiring functional interactions over
287 evolutionary time. In summary, our analyses revealed that evolutionary dynamics of ncORFs have
288 widespread impacts on their translation and potential function.
289
290 **Discussion**
291 Reliable annotation of translated ncORFs is critical for understanding their functional roles and
292 evolutionary significance. In this study, we developed a standardized pipeline for the annotation of
293 translated ncORFs and applied it to both humans and mice due to the extensive publicly available Ribo-
294 Seq datasets. Compared to previous efforts, our annotation framework offers several key improvements.
295 First, we selected only high-quality libraries with sufficient read coverage and acceptable in-frame RPF
296 rates. Our earlier work demonstrated that both library size and in-frame rates impact the reproducibility
297 and accuracy of ncORF predictions[20]. This step helps mitigate false annotations arising from suboptimal
298 data quality. Second, although many previously characterized functional ncEPs are associated with
299 cancer-related ncORFs[1], ncORFs may also have important functions under physiological conditions.
300 Importantly, such ncORFs are more likely to be subject to evolutionary constraints. Therefore, we
301 restricted our analysis to normal cells and tissues, allowing us to identify more biologically relevant and
302 conserved ncORFs. Third, given the variable performance across ncORF detection methods and the low
303 reproducibility of predictions between biological replicates[20], we implemented stringent reproducibility
304 criteria. Specifically, we retained only ncORFs detected by multiple methods and across independent
305 datasets, enhancing the robustness of our final annotation. Finally, we applied a suite of well-validated
306 filtering metrics from prior studies[39-41] to minimize the inclusion of false positives, including those
307 arising from translational noise[59,60] or non-ribosomal contamination. As a result, our annotated ncORFs
308 show significant enrichment for GENCODE ncORFs, especially those with MS evidence or
309 reproducible detection across independent studies. These features collectively underscore the reliability
310 and utility of our annotation for future functional and evolutionary studies of non-canonical translation.
311 However, our catalog may remain incomplete due to the uneven distribution of publicly
312 available Ribo-seq datasets across tissues and cell types. Broader and more balanced tissue coverage
313 will be necessary to generate a more comprehensive and less biased ncORF annotation, which would
314 also enhance the cotranslation analysis. Additionally, our proteomic validation relies on previously
315 compiled sets of MS-supported ORFs rather than de novo reanalysis of raw mass spectrometry datasets.
316 Incorporation of candidate ncORFs into reference proteome databases prior to large-scale, standardized
317 searches of public MS runs would substantially improve detection sensitivity and provide stronger
318 evidence for their translational products[45].

From the perspective of new gene evolution, ncORFs represent a transitional stage between non-coding DNA/RNA and protein-coding genes[61,62]. A major challenge in reclassifying ncORFs as bona fide coding genes lies in determining whether the putative proteins they encode are functionally relevant. Our analysis of ncEPs revealed that most lack recognizable protein domains and are enriched for IDRs. Therefore, we propose that many ncORFs may function in a non-autonomous manner by interacting with other proteins to exert their effects. This hypothesis aligns with prior studies showing physical interactions between ncEPs and known proteins[28,29,34]. Supporting this view, our WGCNA analysis uncovered widespread co-translation between ncORFs and canonical CDSs, suggesting potential interaction partners and functional contexts.

We also assessed whether ncORFs are subject to evolutionary constraints consistent with functional protein coding. Using both population-level and cross-species analyses, we found that a substantial fraction of ncORFs bear signatures of purifying selection, in line with previous studies[33,63-66]. Notably, their conservation profiles exhibit nucleotide-level features characteristic of canonical CDSs, supporting the interpretation that selection acts on the encoded peptide products rather than merely on translational activity per se. Furthermore, we reconstructed the evolutionary trajectories of thousands of ncORFs in both humans and mice and observed sustained coding potential since their origination. Complementing these findings, we identified a subset of ncORFs that preserve intact ORF structures across most descendant species, suggesting ongoing purifying selection against disabling mutations. These lineage-specifically conserved and potentially coding ncORFs likely contribute to clade-specific functional innovation and represent compelling candidates for future functional characterization.

We further explored how evolutionary history shapes ncORF translation across tissues or cell types. Our results indicate that older ncORFs are more broadly and highly translated and have established more extensive co-expression with canonical CDSs. Moreover, when comparing ncORFs of similar age, those that are lineage-specifically constrained consistently exhibit higher translation levels, reinforcing their likely functional importance. Interestingly, we found that younger ncORFs with strong evolutionary constraints tend to have lower tissue specificity in their translation, potentially reflecting a phase of widespread expression that facilitates functional recruitment. In contrast, older ncORFs with positive coding potential tend to display increased tissue specificity, implying functional specialization over evolutionary time. Collectively, these findings support a model in which ncORFs evolve from broadly expressed, weakly functional elements into specialized, conserved effectors—though further empirical work is needed to validate this hypothesis.

In conclusion, we provide a comprehensive annotation of translated ncORFs in humans and mice, offering a valuable resource for functional and evolutionary genomics. Our integrated analysis of sequence evolution and translational landscape sheds light on the evolutionary trajectory and functional integration of ncORFs. The consistency of these patterns in both species suggests that our findings may generalize to other mammals. However, realizing the full biological significance of ncORFs,

356 particularly lineage-specific ones, will require experimental validation. Recent advances in high-
357 throughput screening technologies[19,29,32,67] offer a promising path forward. Lastly, although our study
358 focused on humans and mice due to the availability of high-quality Ribo-Seq data, we anticipate that
359 our annotation will serve as a training resource for machine learning models aimed at predicting
360 translated ncORFs in non-model organisms, thus broadening our understanding of genome coding
361 potential across species.
362
363
364 **Methods**
365 **Quality control and preprocessing of Ribo-Seq libraries**
366 Ribo-Seq libraries from normal human or mouse samples without genetic modifications or perturbations
367 (Table S7) were downloaded from the European Nucleotide Archive (ENA) or the Short Read Archive
368 (SRA). For ncORF annotation, libraries containing fewer than five million reads were excluded.
369 Libraries derived from cancer tissues or cell lines were also removed based on sample metadata or the
370 associated publications. Next, adapter sequences and any random nucleotide additions were trimmed
371 using Cutadapt (v3.7)[68] and reads shorter than 18 nucleotides were discarded. Remaining reads were
372 aligned to the human or mouse reference genome (GRCh38 or GRCm39, ENSEMBL[69] release 107)
373 using the STAR aligner (v2.7.10a)[70]. To assess library quality, the proportion of in-frame ribosome-
374 protected fragment (RPF) reads was calculated using the PSite package[71]. Libraries with ≥60% in-frame
375 RPFs were classified as high quality. Only samples containing at least two high-quality libraries were
376 retained for ncORF annotation (Table S1). For the expression analyses, all the downloaded Ribo-Seq
377 libraries were used to cover more samples.
378
379 **ncORF annotation**
380 For each Ribo-Seq library, translated ORFs initiating with AUG, CUG, GUG, or UUG and encoding at
381 least five amino acids were predicted using PRICE[36], RiboCode[37], and Ribo-TISH[38], as described
382 previously[20]. To define novel ORFs, annotated CDSs and their truncation or extension variants (i.e.,
383 ORFs with the same start or stop position as annotated CDSs) were excluded. To eliminate likely false
384 positives resulting from non-ribosomal contamination or stochastic molecular errors, we calculated
385 RRS[40,41] and FLOSS[39] of each ncORF. RRS measures the drop in RPF coverage after stop codon—a
386 key signature of active translation beyond three-nucleotide periodicity[40,41], whereas FLOSS assesses
387 whether the RPF length distribution resembles that of canonical CDSs, helping to distinguish authentic
388 ORFs from non-ribosomal signals[39]. In each library, uniquely mapped RPFs were assigned to the
389 corresponding P-site, and genome-wide P-site coverage was computed with the PSite package. Similar
390 to previous studies[40,41], RRS was calculated as the sum of coverage across the five codons preceding
391 the stop codon divided by the sum across the five trinucleotides following the stop codon. FLOSS was
392 calculated by first deriving a reference RPF length distribution vector (P_{ref}) using all RPFs uniquely

mapped to annotated CDSs. For each ORF (ncORF or CDS), a read length frequency vector (P_{ORF}) was computed, and FLOSS was calculated as $\sum|P_{ref} - P_{ORF}|/2$. The empirical upper bound for acceptable FLOSS was set using Tukey's method (i.e., the third quartile + 1.5 × inter-quartile range of FLOSS values across all CDSs)[39]. ncORFs with fewer than 10 P-site-mapped RPFs, RRS ≤ 1, or FLOSS values exceeding the empirical upper bound were filtered out.

Integration of ncORF prediction across different libraries of the same species was nontrivial due to extensive overlap among ncORFs in genomic coordinates. To address this issue, we used a graph-based clustering approach. We created a graph where two ORF vertices were connected if they share the same start or stop codon. Then, each set of connected ncORFs in the graph represents a cluster of overlapping ncORFs. To ensure reproducibility, only clusters identified by ≥2 methods in ≥2 libraries were retained, yielding 13,071 clusters in humans and 17,674 clusters in mice. To ensure compatibility with the latest reference gene annotations (ENSEMBL release 111), we removed any ncORFs that were newly classified as annotated CDSs. A manual inspection of RPF signals using the GWIPS-viz Ribo-Seq track[72] in the UCSC Genome Browser[73] revealed additional spurious ncORFs overlapping in-frame with CDSs from other transcripts. These were likely misidentified due to shared translation signals and were conservatively excluded using custom scripts, leaving 11,623 clusters in humans and 16,485 in mice.

To facilitate downstream analyses, a representative ncORF was selected for each cluster using a two-step strategy. First, a representative transcript was selected by prioritizing those with matched annotation from NCBI and EMBL-EBI (MANE)[42] (for humans) or APPRIS principal[74]; otherwise, the longest transcript was chosen. Then, among ncORFs within the representative transcript, a single ncORF was selected based on start codon hierarchy (AUG > CUG/GUG > UUG) following empirical evidence[28,36]. If multiple ncORFs shared the same preferred start codon, the longer one was chosen.

For validation, our ncORFs were compared with previously annotated GENCODE ncORFs[2] and those with MS evidence compiled previously[20]. Tier annotations of GENCODE ncORFs were obtained from a recent study[45]. Known functional ncORFs and their genomic coordinates were manually curated from published literature (Table S4). To account for possible proteoforms arising from alternative initiation and splicing, we considered an ncORF to be recovered if either its start or stop codon was identical to any ncORF in our annotation.

ncORF sequence analyses

The secondary structure surrounding each ORF (ncORF or CDS) start codon was predicted with RNAfold[75] on a 150 nt window extracted from the transcript in which the ncORF resides starting from the 30th nucleotide upstream of the start codon. For each position within this window, the overall pairing probability was calculated by dividing the number of ORFs with a base-paired residue at that position by the total number of ORFs analyzed. The tRNA adaptation index and effective number of codons for

429 ncORFs and CDSs were calculated using the "cubar" R package[76]. tRNA gene annotations in humans
430 and mice were obtained from GtRNAdb (release 22)[77].
431
432 **Putative ncEPs analyses**
433 Known domains within ncEPs were identified using InterProScan (v5.64-96.0)[78] with default
434 parameters. Repetitive sequence annotations were downloaded from the UCSC Table Browser.
435 BEDTools (v2.31.1)[79] was used to identify TE-overlapping ncORFs. IDRs were predicted with
436 IUPred3[80] with analysis type set to "short disorders" and smoothing disabled. For each ORF, residues
437 with disorder tendency score > 0.5 were considered disordered, and the IDR fraction was calculated as
438 the proportion of disordered residues relative to the ORF length.
439
440 **Gnocchi Score and PhyloP of ncORFs and CDSs**
441 Genome-wide Gnocchi scores computed using 1 kb sliding windows (with 100bp step size) were
442 downloaded from the gnomAD database[50]. For each base pair, the Gnocchi score was defined as the
443 average across the ten overlapping 1kb windows that covered that position using BEDTools. The final
444 Gnocchi score for each ORF (ncORF or CDS) was obtained by averaging the scores of all base pairs
445 within the ORF.
446 Base-wise PhyloP scores computed across 441 mammals or 239 primates[51] were downloaded
447 from the UCSC Genome Browser. For each ncORF or CDS, PhyloP values were extracted for a ±30 bp
448 window surrounding the start and stop codons using custom scripts. These values were then averaged
449 over different ORFs to generate metagene profiles for visualization. To evaluate three-nucleotide
450 periodicity, the metagene profiles were subjected to autocorrelation analysis using the "ac.test" function
451 from the "testcorr" R package[81]. Statistical significance was assessed using the robust $\tilde{t}$ statistics.
452
453 **ncORF origination node and mode**
454 Whole genome alignments and corresponding species trees were downloaded from the UCSC Genome
455 Browser (GRCh38 100-way for humans and GRCm39 35-way for mice). The origination node and
456 mode for each ncORF were inferred following previous approaches[33,34]. For each ORF, the sequence
457 from the reference genome and its orthologous regions in other species were extracted. Orthologous
458 sequences covering less than 50% of the ncORF length relative to the reference species were excluded.
459 The remaining sequences were realigned with MAFFT (v7.520)[82] with default parameters. The
460 corresponding gene tree was derived by pruning the species tree using Gotree (v0.4.3)[83] with default
461 settings. Ancestral sequence reconstruction was performed using FastML (v3.11)[84] with parameters "--
462 seqType nuc --SubMatrix HKY --OptimizeBL no --indelReconstruction ML". An ORF structure was
463 considered intact in an orthologous or ancestral sequence if one of the first three codons was a start
464 codon and at least 70% of the 5' region of the reference ncORF was free of in-frame stop codons. The
465 origination node was defined as the most ancient ancestral node that retained an intact ORF structure.

466 An ncORF was classified as *de novo* if the origination node had an ancestor covering at least 50% of
467 the ncORF sequence but lacking an intact ncORF structure. Otherwise, the origination mode was
468 classified as uncertain. The distance from an ancestral node to the reference species (tip node) was
469 calculated by summing branch lengths along the connecting path using the ape R package (v5.8)[85].
470
471 **ncORF BLS and PhyloCSF**
472 For each ncORF, global BLS was calculated as the ratio of the total branch length of the subtree
473 connecting all leaves with intact ORF structures to the total branch length of the full species tree as in
474 our previous study[48] with custom scripts. Local BLS was calculated similarly, but the denominator was
475 replaced by the total branch length of the subtree defined by the origination node and all of its
476 descendant nodes (including leaves). To evaluate coding potential, the alignment of all species
477 descending from the origination node was converted into Multiple Alignment Format after removing
478 the stop codon. PhyloCSF scores were then computed using the "score-msa" module of PhyloCSF++[86].
479
480 **ncORF expression analysis**
481 To quantify translation levels of ncORFs and CDSs in Ribo-Seq libraries, we assigned uniquely mapped
482 reads to corresponding P-site positions with the PSite package. The read count for each ORF was
483 calculated as the total number of RPFs whose P-sites fell within the ORF. To reduce bias caused by RPF
484 enrichment at ORF termini, reads mapping to start or stop codons were excluded. For CDSs, only the
485 count of the representative transcript of each gene as defined above was used for downstream analyses.
486 For samples with multiple libraries, read counts were aggregated prior to normalization. Sample-
487 specific size factors were estimated using CDS counts and applied to normalize both ncORF and CDS
488 counts across samples using DESeq2[87]. RPKM for an ORF was calculated as normalized read counts /
489 ORF length (nt) / library size × 10^9, where library size was the median of total read counts across
490 samples to ensure cross-sample comparability. An ORF (ncORF or CDS) was considered translated if
491 its RPKM was ≥ 1. To evaluate tissue specificity of ncORF translation, the normalized RPF count X_k
492 of an ORF in sample k was log-transformed as $x_k = log(X_k + 1)$. Then, tissue specificity index[88] was
493 calculated as $\tau = \sum_k^n (1 - \tilde{x}_k)/(n-1)$, where $\tilde{x}_k = x_k / \max_{1 \le j \le n} x_j$ and n is the total number of samples.
494 To quantify RNA levels, raw reads of deep transcriptome profiling across different human or mouse
495 tissues from previous studies[55,56] were downloaded from SRA. Gene expression levels (transcript per
496 million, TPM) were estimated using salmon (v1.10.3)[89] with parameters "--seqBias --gcBias --posBias
497 -l A".
498 WGCNA analysis[58] of ncORF and CDS translation was performed using the PyWGCNA
499 package (v2.0.5)[90] with default settings. Only ORFs with normalized RPF counts ≥ 10 in more than half
500 of the samples were included. The soft threshold was determined automatically by PyWGCNA. Due to
501 differences in soft thresholds, two ORFs were considered cotranslated if their topological overlap > 0.1

(human) or > 0.01 (mouse).[58] To test whether co-translation is enriched between ncORFs and CDSs from the same transcript, gene identities of CDSs were randomly permuted among all co-translated ncORF–CDS pairs. This procedure was repeated 1,000 times, and the empirical *P* value was calculated as $(m + 1) / 1{,}000$, where m is the number of permutations in which the number of same-gene pairs was greater than or equal to the observed count. The bipartite network of ncORF-CDS co-translation was clustered using the Leiden algorithm[91] implemented in igraph (v2.1.3)[92] by optimizing modularity with a resolution of 0.2. The top 20% of nodes by betweenness centrality were designated as hub nodes. Gene ontology analysis of hub genes in the largest modules was conducted using ClusterProfiler (v4.12)[93].

Code availability

All analyses were primarily conducted using R software (v4.3). Unless otherwise stated, statistical tests are two-sided. The code is available at https://github.com/gxelab/ncorf_mammals and custom scripts can be found at https://github.com/gxelab/scripts.

Data availability

Ribo-Seq libraries for ncORF annotation were obtained from the ENA or SRA, with accession codes listed in Table S1. Libraries used for ncORF and CDS translation quantification were listed in Table S7. RNA-Seq libraries for gene expression profiling across different human or mouse tissues were from previous studies[55,56] and are available with ENA accession numbers E-MTAB-2836 and E-MTAB-10276, respectively. Whole genome alignment across vertebrates, annotation of genomic repetitive elements, and base-wise PhyloP in primates and mammals[51] were downloaded from the UCSC Genome Browser[73]. Gnocchi scores were downloaded from gnomAD[50]. All the other data were presented in the manuscript or supplementary tables and are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

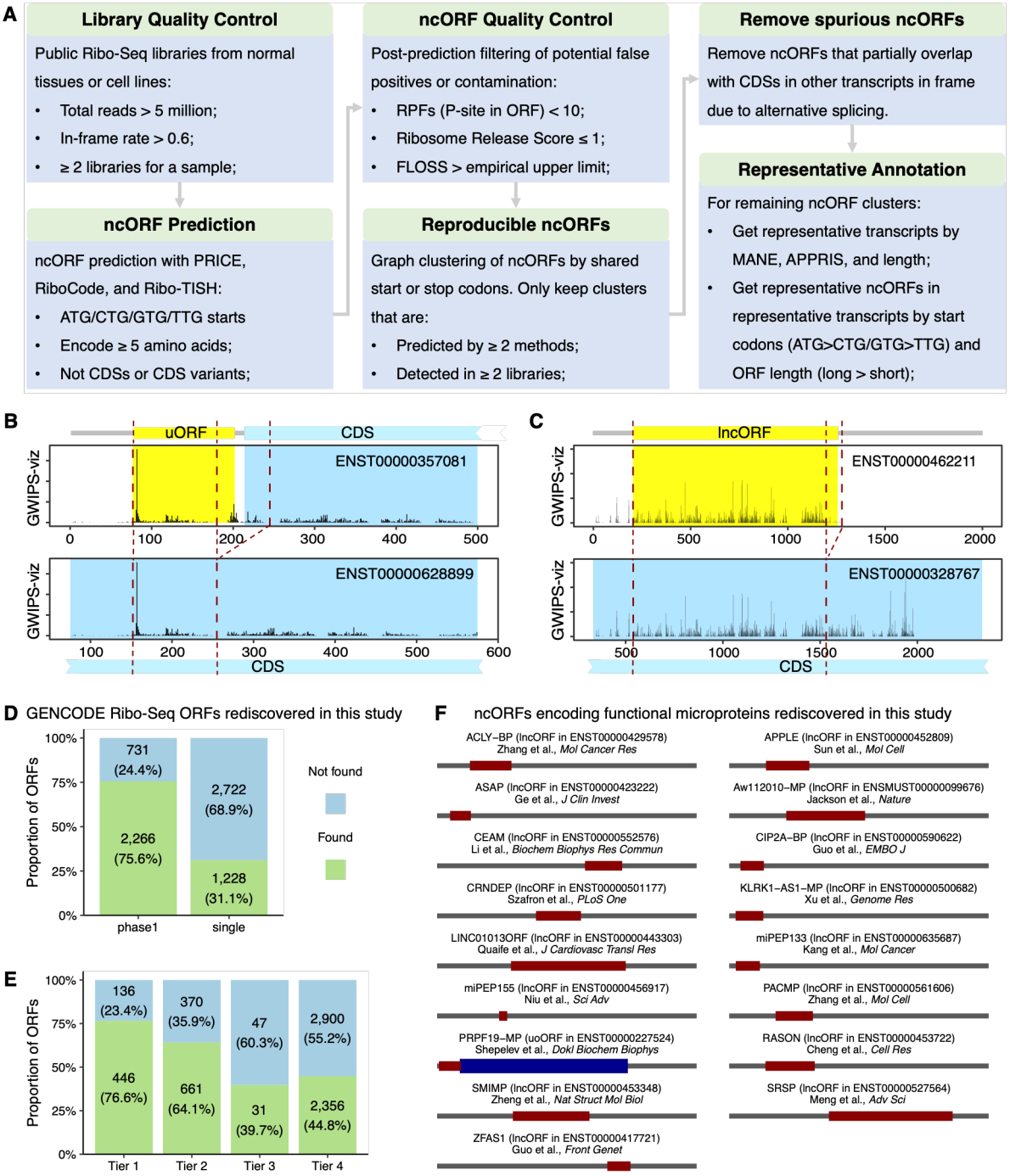


Figure 1. Annotation of ncORFs in humans and mice.

(**A**) Overview of the integrative pipeline for ncORF annotation in human and mouse genomes. (**B-C**) RPF coverage plot for a representative uORF (B) and a lncORF (C), visualized using the Ribo-Seq signal track from GWIPS-viz[72]. ncORFs were shown in yellow and CDSs in cyan. Dashed lines indicate identical genomic regions shared across transcript isoforms. (**D-E**) Proportion of GENCODE ncORFs rediscovered in this study. GENCODE ncORFs were stratified either by reproducibility across independent studies (D) or by tiered translation evidence strength (E). (**F**) Known ncORFs that were experimentally characterized in earlier studies and independently rediscovered in this study from

humans and mice. CDS, coding sequence; FLOSS, fragment length organization similarity score; MANE, matched annotation from NCBI and EBI; ncORF, non-canonical open reading frame; ORF, open reading frame; RPF, ribosome-protected fragment.

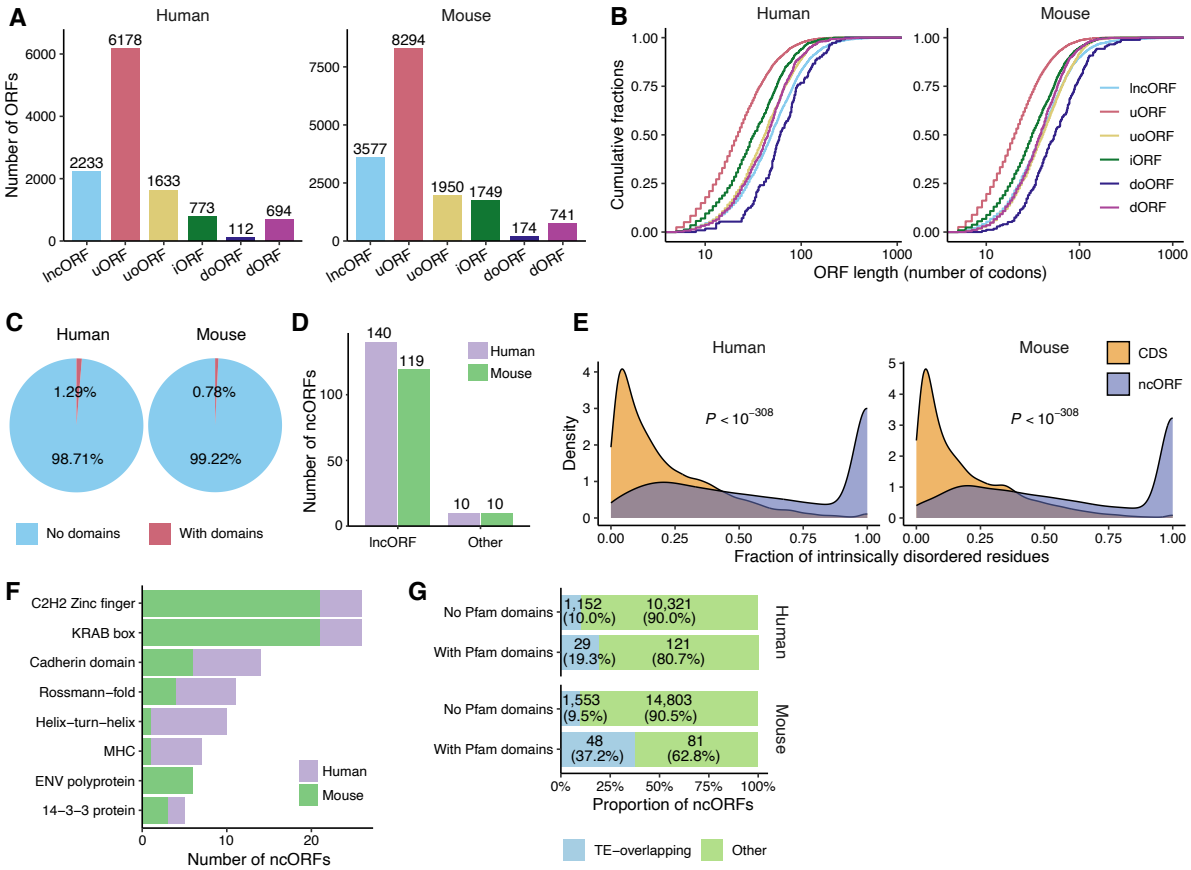


Figure 2. Sequence features of ncORFs and putative ncEPs in humans and mice. (**A**) Number of ncORFs belonging to different categories. (**B**) Distribution of ncORF lengths. (**C**) Proportion of ncEPs that contain known Pfam domains. (**D**) Number of ncORFs with Pfam domains among lncORFs and other ncORF categories. (**E**) Distribution of the proportion of intrinsically disordered residues in CDS- and ncORF-encoded proteins. (**F**) Most frequently identified Pfam domains among human and mouse ncORFs. (**G**) Proportion of ncORFs overlapping with TEs, stratified by presence or absence of Pfam domains. Differences were assessed using Wilcoxon rank-sum tests. CDS, coding sequence; ncORF, non-canonical open reading frame; ncEP, ncORF-encoded protein; TE, transposable element.

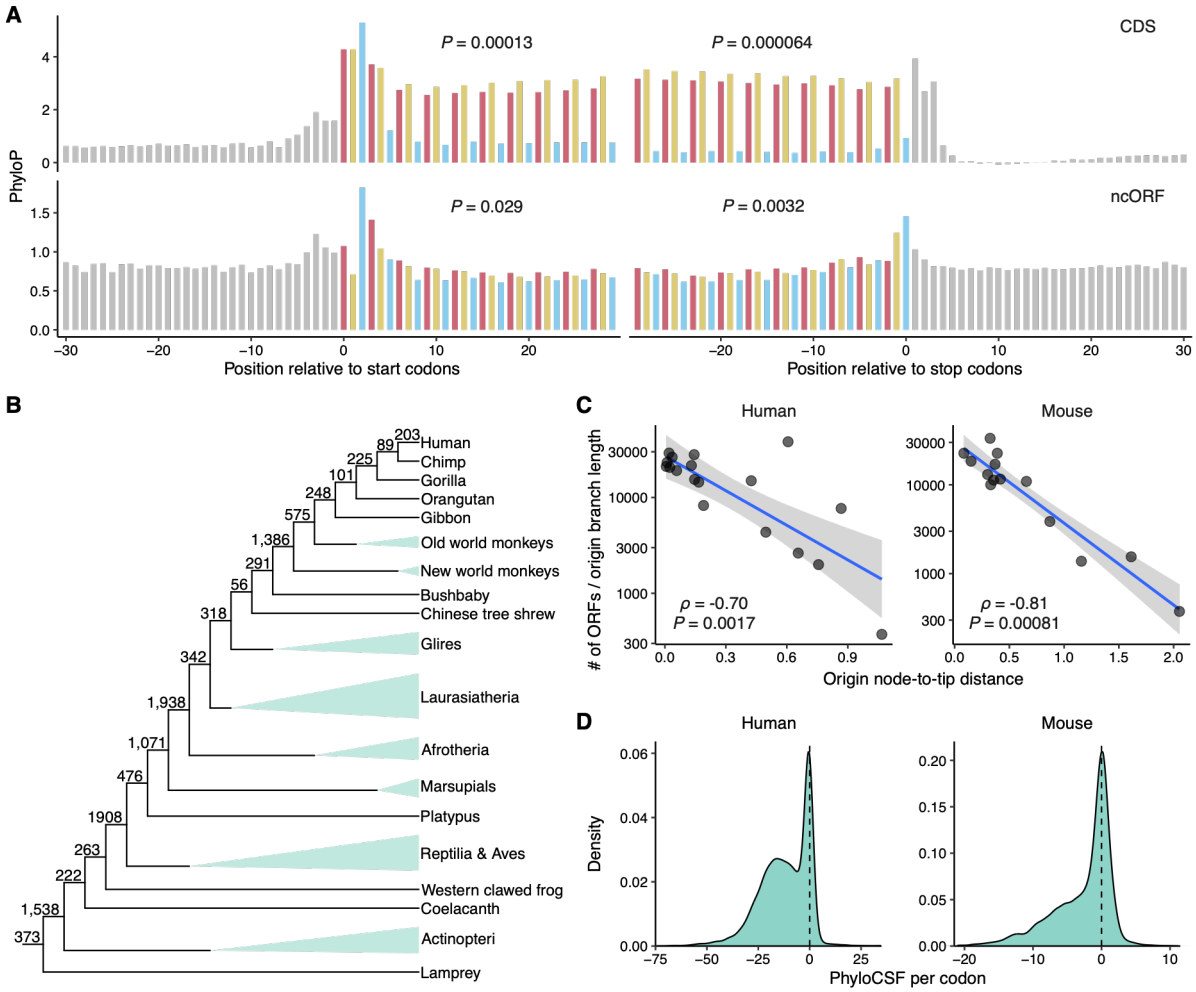
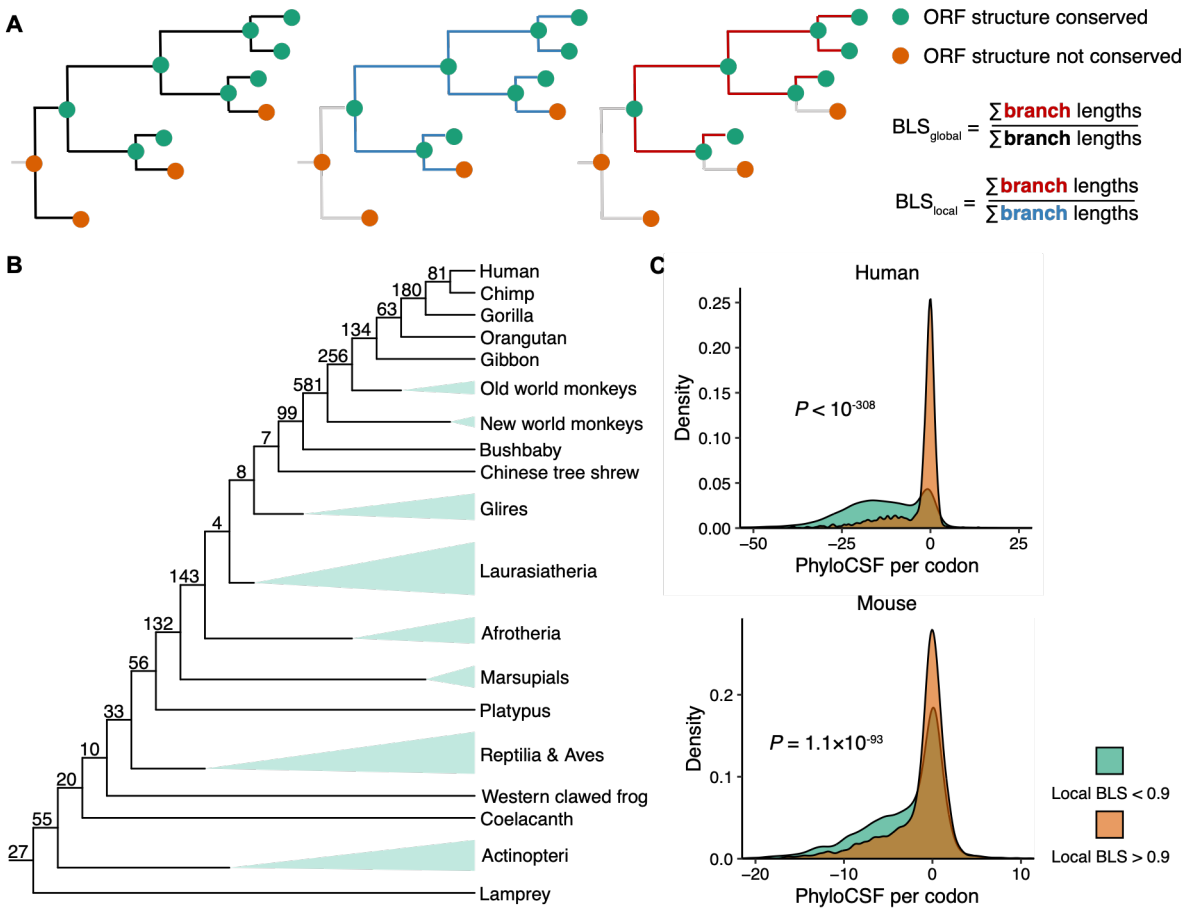


Figure 3. Evolutionary constraints of ncORFs.

(**A**) The mean PhyloP scores of 30 base pairs upstream and downstream of the ORF start or stop codons in mammals. Codons were delineated with bars of alternating colors by different frames, and nucleotides in untranslated regions were shown in grey. The significance of three-nucleotide periodicity was assessed by autocorrelation with a lag of three. (**B**) Cladograms of vertebrates with the number of ncORFs originating at each ancestral branch of humans. Triangles indicate species merged into larger clade for visual simplicity. (**C**) Relationship between ORF origination rates and node ages measured as node-to-tip distances. Origination rate was defined as the number of ncORFs that originated on a branch divided by the branch length. Blue lines show linear regression fits, and grey bands represent 95% prediction confidence intervals. Spearman's correlation is indicated. (**D**) Distribution of ncORF PhyloCSF scores normalized by the number of codons in per ncORF. The dashed line denotes zero. CDS, coding sequence; ncORF, non-canonical open reading frame; ORF, open reading frame.



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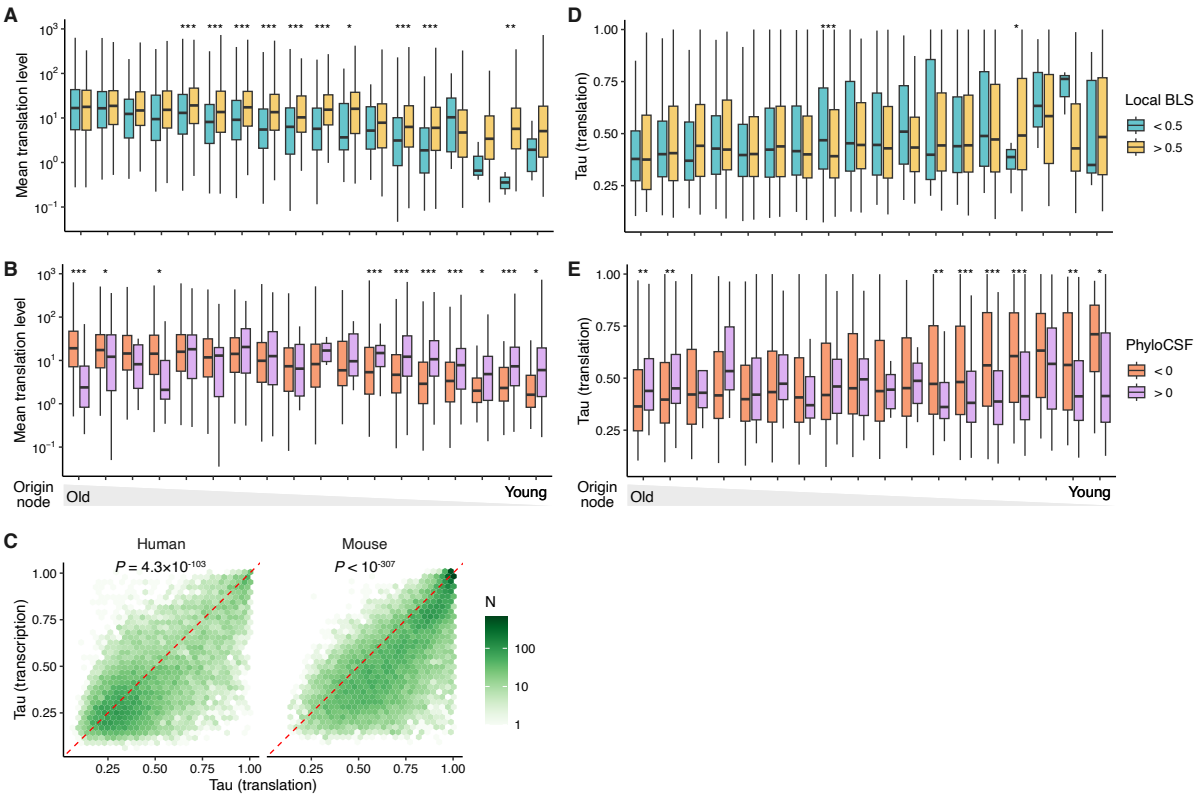
846 **Figure 4. Lineage-specifically conserved ncORFs.**

847 (**A**) Schematic illustration for BLS calculation. (**B**) Cladograms of vertebrates showing the number of
848 lineage-specifically conserved ncORFs (local BLS > 0.9) at each ancestral node for humans and mice.
849 (**C**) Distribution of PhyloCSF scores per codon for lineage-specifically conserved ncORFs compared
850 to all other ncORFs. BLS, branch length score; ncORF, non-canonical open reading frame; ORF, open
851 reading frame.



852

853 **Figure 5. Evolutionary dynamics of ncORF expression.**

854 (**A**) Distribution of mean translation levels of human ncORFs grouped by their origin nodes and further
855 stratified by local BLS. Statistical significance was assessed using Wilcoxon rank-sum tests. ***, $P <$
856 0.001; **, $P < 0.01$; *, $P < 0.05$. (**B**) Similar to (A) but ncORFs are further stratified by local BLS. (**C**)
857 Relationship between ncORF tissue specificity at translation and transcription levels. Differences were
858 determined with Wilcoxon signed-rank tests. (**D-E**) Similar to (A) and (B) but showing the distribution
859 of tissue specificity at the translation level. CDS, coding sequence; ncORF, non-canonical open reading
860 frame; ORF, open reading frame.

861

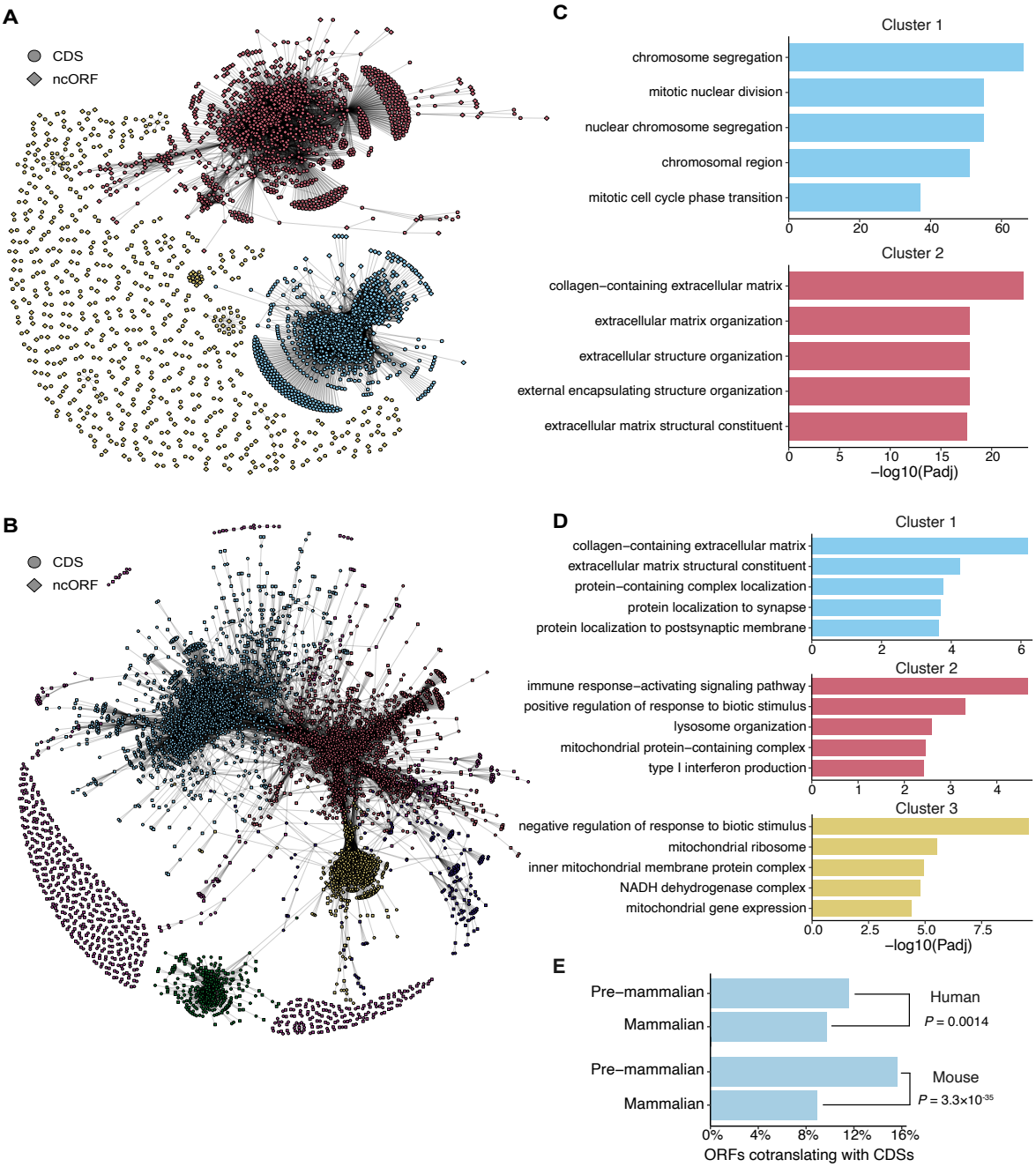


Figure 6. ncORF-CDS co-translation network.

(**A**) Bipartite network of ncORF-CDS co-translation in humans. (**B**) Bipartite network of ncORF-CDS co-translation in mice. ncORFs and CDSs are represented by different shapes and colored according to their cluster membership. Only the largest clusters were highlighted (top two in humans and top five in mice). (**C**) Top five enriched gene ontology terms for each of the two largest clusters in the human network shown in (A). (**D**) Top five enriched gene ontology terms for each of the three largest clusters in the mouse network shown in (B). (**E**) Proportion of ncORFs cotranslating with CDSs among ancient (pre-mammalian origin) versus younger (mammalian-specific) ncORFs. Differences were tested with Fisher's exact tests. CDS, coding sequence; ncORF, non-canonical open reading frame; ORF, open reading frame.