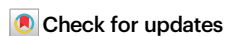


Deciphering tRNA repertoires and translation coordination during mouse early embryogenesis by ORACLE-tRNAseq

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Translational control is vital during the maternal-to-zygotic transition (MZT), yet the landscape of embryonic transfer RNA (tRNA) pools has been difficult to explore. Here, we develop Optimized Reaction for Accurate Capture of Low-input Entities tRNA Sequencing (ORACLE-tRNAseq), enabling robust tRNA profiling from as few as five mouse oocytes. We map tRNA landscapes from oocyte to blastocyst, identifying a distinct transition to embryonic tRNA repertoires and upregulation of tRNA pseudogenes at the 4-cell stage. Integrated multi-omics analyses reveal that zygotic tRNA gene activation coincides with zygotic genome activation (ZGA) and correlates with H3K4me3 establishment and chromatin remodeling. By coupling ORACLE-tRNAseq with Riboseq, we demonstrate that embryonic tRNA anticodon pools coordinate with high translation-efficiency gene pools to preferentially establish the zygotic translation machinery, particularly from major ZGA onwards. Collectively, these findings provide a resource for understanding translational regulatory networks during early embryogenesis.

Early mammalian embryonic development from the one-cell zygote to the multicellular blastocyst is a closely orchestrated and dynamically regulated process, characterized by dramatic epigenetic reprogramming and intricate gene regulatory networks¹. A critical developmental switch during this period is the maternal-to-zygotic transition (MZT), which marks the onset of embryonic gene expression control². Using ultra-trace multi-omics technologies, researchers have uncovered the dynamics of transcripts, mRNA translation and proteomic landscape during mammalian preimplantation embryonic development^{3–9}, revealing the dynamic regulation of maternal mRNA decay and zygotic mRNA replacement, along with a shift in translational regulation and embryonic protein synthesis to further adapt to rapid cell division and

cell fate establishment after implantation. Notably, it also uncovered the divergent dynamics among transcripts, ribosome binding and protein outputs in mammalian embryos^{6–8}, emphasizing the importance of developing a global overview of translation coordination networks to enhance our understanding of embryonic development.

Translational regulation involves the coordination among post-transcriptional modifications, ribosome binding and transfer RNA (tRNA) decoding capacity. As a carrier of amino acids that base-pair with mRNA codons, tRNA is essential for translation. Contrary to the earlier view of tRNA as a ubiquitous housekeeping molecule, studies have revealed distinct tRNA expression patterns across different organs^{10,11}, and identified specialized tRNA repertoires in differentiated

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and proliferating cell types, indicating indispensable regulatory roles of tRNA in translational control¹². However, the coordination between cellular tRNA supply and the codon demand for cellular translation in mammalian embryogenesis is still under investigation. Recently, two groups deciphered the dynamic regulation of tRNA landscape during zebrafish embryonic development, uncovering a gradual switch between maternal and zygotic tRNA pools^{13,14}. Interestingly, the insufficient supply of tRNA can lead to the degradation of maternal mRNAs enriched in slowly decoded codons during zebrafish MZT¹⁴. However, the dynamic landscape and translational regulatory roles of tRNAs in early mammalian embryonic development remain unclear.

In recent years, various tRNA sequencing technologies have been developed, including next-generation sequencing (NGS) based strategies, such as ARM-seq¹⁵, DM-tRNAseq¹⁶, mim-tRNAseq¹⁷, Induro-tRNAseq¹⁸, ALL-tRNAseq¹⁹, hydro-tRNAseq²⁰, and nanopore or mass spectrometry (MS) based direct-tRNA sequencing methods, like MLC-Seq²¹, Nano-tRNAseq²² to characterize the heterogeneity of cellular tRNA repertoires. These protocols typically require a large starting amount of total RNA in the microgram range or hundreds of nanograms of gel-purified tRNA-enriched RNA fractions, and involve multiple RNA recovery steps and various independent reaction systems. Therefore, despite demonstrating good performance on tRNA profiling, these protocols are still not feasible for studying scarce biological materials such as mammalian early embryos.

To address the challenge of quantifying tRNA pools in minute mammalian embryos, we developed Optimized Reaction for Accurate Capture of Low-input Entities tRNA Sequencing (ORACLE-tRNAseq), a robust method for comprehensive tRNA profiling from limited sample input. Using this approach, we map the dynamic landscape of tRNA expression and modification during mouse early embryonic development, revealing the switch signature of tRNA pools during the MZT. Furthermore, by integrating tRNA expression data with multi-omics datasets (ATAC-seq²³, ChIP-seq²⁴, nascent RNA-seq and ribosome profiling⁴), we analyze the transcriptional regulation of zygotic tRNA genes and discover translational coordination between tRNA pools and high-translation-efficiency gene pools. These findings provide a comprehensive understanding of the roles played by tRNA repertoires during early embryonic development.

Results

Development of an ultra-low-input tRNA sequencing method

To enable robust NGS-based profiling of tRNA molecules from ultra-low input samples, we established a method termed ORACLE-tRNAseq (Fig. 1a). This method starts with cell lysates and completes library construction in a single-tube reaction. ORACLE-tRNAseq employs a ligation-free strategy by adding a poly(A) tail to the 3'-OH end of tRNA molecules using Poly(A) polymerase, followed by reverse transcription and template switching to add a Template Switching Oligonucleotide (TSO) containing Unique Molecular Identifiers (UMIs) to the 5' end of tRNA molecules, resulting in UMI-tagged cDNA molecules containing the TSO complementary sequence. We confirmed that T4PNK is not essential to repair the tRNA 3' end for enhancing the ligation efficiency of the poly(A) tail after removing the amino acid (Supplementary Fig. 1a). We performed bioinformatic analysis using the mim-tRNAseq computational pipeline¹⁷ and limited the analysis to reads of 50 nt and longer to exclude tRNA-derived small RNAs (tsRNAs). The mim-tRNAseq computational pipeline has been shown to exhibit superior alignment performance for tRNA libraries compared to general-purpose alignment software such as Bowtie and Bowtie2¹⁷. Furthermore, this pipeline offers a comprehensive range of downstream analysis categories.

Because low-input strategies require higher PCR cycle number, which may introduce bias during amplification, we evaluated the effectiveness of incorporating UMI technology in the ORACLE-tRNAseq workflow. We compared libraries before and after UMI-

based deduplication, and the results showed that a substantial number of PCR duplicates were removed in both 100 pg and 1 ng mESC total RNA libraries (Fig. 1b).

To confirm that duplicate removal was unbiased, we compared the 1 ng input library before and after deduplication with a higher-input sample (Fig. 1c). After UMI-based deduplication, the R^2 values for the $y=x$ comparison with the higher-input sample increased substantially (from 0.6420 and 0.7175 to 0.8632 and 0.9202), highlighting the importance of UMI technology in low-input pipelines. Following UMI-based deduplication, the 100 pg versus 100 ng input samples showed good correlation (Spearman correlation coefficients > 0.90) and faithfully recapitulated differences observed with 1 ng liver total RNA input (Fig. 1d). However, we observed substantial loss of low-abundance tRNAs in the 100 pg input library (Fig. 1e), suggesting that using at least 1 ng of total RNA in ORACLE-tRNAseq can markedly improve sequencing quality.

We further evaluated the differences between starting from cell lysates and total RNA input, and the results showed high consistency (Spearman correlation coefficient = 0.9873, Fig. 1f). Libraries constructed using ORACLE-tRNAseq exhibited high coverage (Median > 80%) (Fig. 1g, Supplementary Fig. 1b) and a high proportion of mature tRNA molecules (Median > 95%) (Supplementary Fig. 1c). We mapped approximately 18.6–38.7% of the total reads to tRNA molecules, with higher tRNA alignment rates observed for libraries from 10 ng and 1 ng input samples (Supplementary Fig. 1d). Further analysis of non-tRNA reads within the libraries (Supplementary Fig. 1e) revealed that they were mainly from rRNA, snoRNA, and mRNA species (Supplementary Fig. 1f). Given that the lengths of rRNA and mRNA are generally greater than the analyzed range, these rRNA and mRNA reads likely represent RNA fragments derived from them²⁵ or degradation products.

To assess differences in absolute quantification between ORACLE-tRNAseq and other tRNA sequencing methods, we compared the HEK293T library generated by ORACLE-tRNAseq with HEK293T libraries from four other methods (mim-tRNAseq, ALL-tRNAseq, Induro-tRNAseq, and hydro-tRNAseq). We found that ORACLE-tRNAseq shows high correlation with all four methods (Spearman's $r=0.8-0.9$, Supplementary Fig. 1g). We also conducted a small-scale spike-in experiment. We synthesized Arg-CCT-2 containing an m¹G modification at position 9 and Met-CAT-1 containing an m¹A modification at position 58, and spiked them in at defined molar ratios before library preparation and sequencing. The observed sequencing read proportions closely matched the input ratios ($r=0.99$, Supplementary Fig. 1h). Meanwhile, we confirmed the reliability of ORACLE-tRNAseq in revealing the true abundance differences of selected tRNA between mESCs and liver using Northern blot validation (Fig. 1h). Next, we applied ORACLE-tRNAseq to mouse oocytes and preimplantation embryos to decipher the tRNA landscape during embryogenesis. Using five oocytes/embryos per group (with an estimated total RNA amount exceeding 1 ng) as input, we successfully decoded the tRNA landscape for nine groups: including two stages of oocytes (GV, MII) and seven stages of embryos (zygote at PN5 stage, early 2-cell (E2C), late 2-cell (L2C), 4-cell (4C), 8-cell (8C), Morula, and Blastocyst) (Fig. 1i), with three biological replicates for each group. The sequencing data showed high coverage of full length tRNAs (Median > 70%) (Supplementary Fig. 1i) and high proportion of CC ends (Supplementary Fig. 1j) in each group. In short, we successfully established an ultra-low input tRNA sequencing method with robust detection capacity to achieve successful mapping of the tRNA repertoire for mouse oocytes and preimplantation embryos using ORACLE-tRNAseq, providing a comprehensive view of tRNA dynamics during early development.

ORACLE-tRNAseq revealed stage-specific tRNA landscapes before and post ZGA

To assess the overall tRNA abundance alteration during embryogenesis, we applied spike-ins to quantify the tRNA pool abundance per

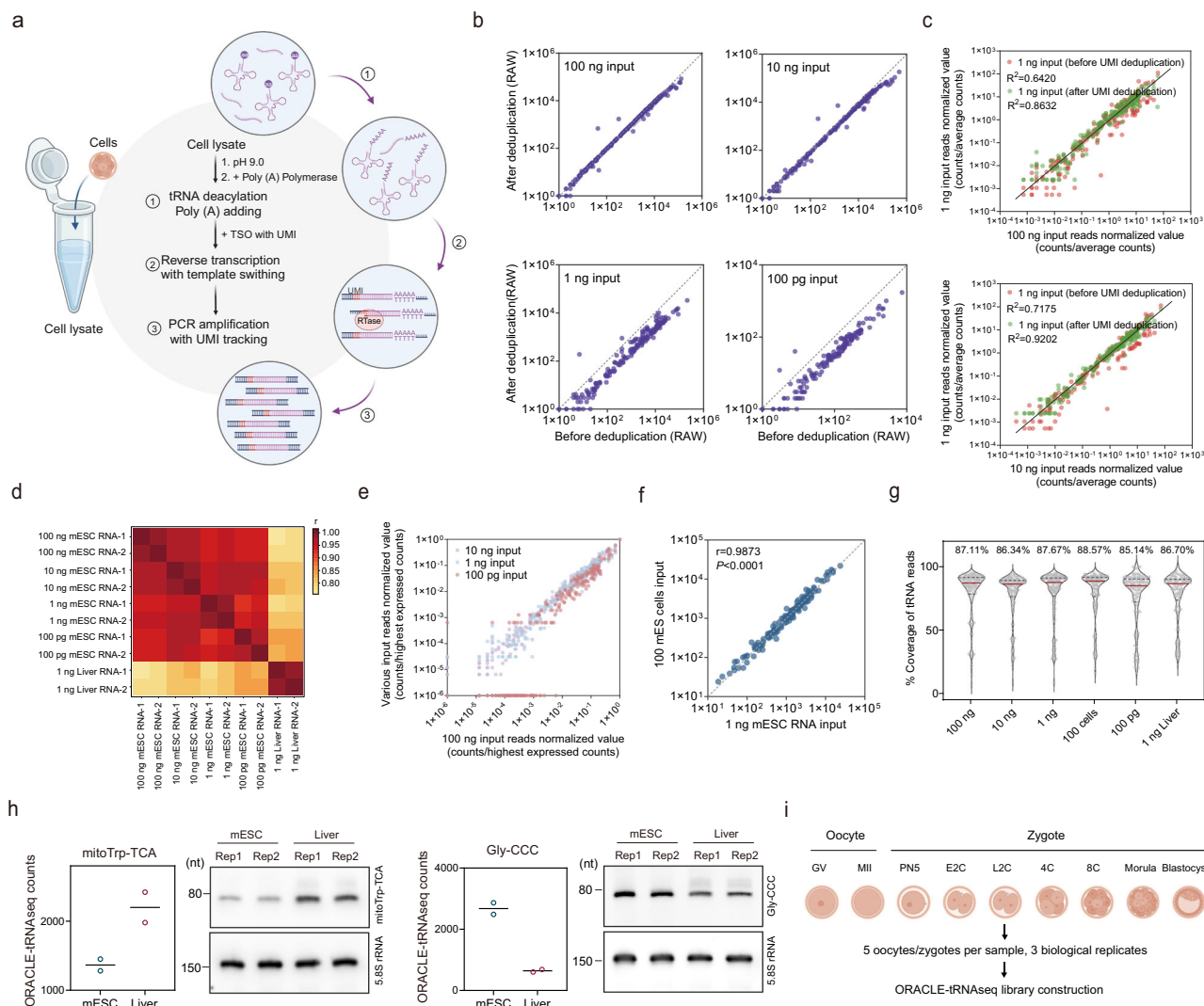


Fig. 1 | Methodology establishment and performance evaluation of ORACLE-tRNAseq. **a** Workflow of ORACLE-tRNAseq library construction, created in BioRender. Li, Y. (2026) <https://BioRender.com/5gb2x7g>. **b** Comparison of RAW reads of tRNA transcripts before and after UMI-based PCR duplicate removal. Each dot represents a tRNA isodecoder. **c** Correlation of 1 ng input samples before and after UMI deduplication compared with higher-input samples (10 ng and 100 ng). Each dot represents a tRNA isodecoder. The regression line is $Y = X$. **d** Spearman correlation of libraries generated from 100 ng to 100 pg mESC total RNA input and 1 ng mouse Liver total RNA input. **e** Comparison of sequencing results across different input amounts. Each dot represents a tRNA isodecoder. **f** Spearman correlation of

libraries generated from 1 ng mESC total RNA input and 100 mESC cell input. P values were calculated using a two-sided Spearman correlation test. **g** Sequencing coverage of tRNA reads at libraries generated from different inputs. Each dot represents a tRNA isodecoder. **h** Northern blot validation of tRNA abundance differences measured by ORACLE-tRNAseq. 5.8S rRNA was used as an internal reference. Each replicate ($n = 2$) represents an independent biological sample or cell culture. Rep, Replicate. **i** Mouse oocytes (GV, MII) and zygotes (PN5, E2C, L2C, 4C, 8C, Morula, Blastocyst) were collected, created in BioRender. Li, Y. (2026) <https://BioRender.com/6nliyk> (**b**, **c**, **e–h**). Source data are provided as a Source Data file.

embryo in each oocyte or embryo stage (Fig. 2a). The results showed that the total tRNA pools had similar abundance among GV and MII oocytes, as well as the zygote and early 2-cell embryo, while it decreased at the L2C stage, then gradually increased during embryonic cleavages and exhibited a noticeable increase at the blastocyst stage, reaching approximately 1.7-fold of the GV stage by the blastocyst stage. Analyzing this together with the normalized ratio plot (Supplementary Fig. 2a), we observed that the increase in the mitochondrial tRNA ratio at the L2C stage was mainly due to a decrease in the abundance of nuclear-encoded tRNAs. Subsequently, the abundance of nuclear-encoded tRNAs increases at a faster change than the increase in mitochondrial tRNA abundance, leading to an increase in the ratio of nuclear-encoded tRNAs. Furthermore, we observed a higher proportion of nuclear-encoded tRNAs in mESCs and MEFs compared to embryos. To facilitate further comparison of the

abundance of different tRNA types across stages, we subsequently performed normalization analysis of tRNA reads across all stages using DESeq2. We performed principal component analysis (PCA) (Fig. 2b) for nuclear-encoded tRNA and mitochondrial tRNA. Both of them showed distinct expression patterns during early development. Specifically, PC1 explained 58.42% of the variance in nuclear-encoded tRNA transcripts and distinguished tRNA transcripts before the first cleavage (GV, MII, PN5) from those after the first cleavage (E2C, L2C). The expression patterns of tRNA transcripts during subsequent cleavages and the period of lineage specification gradually became closer to those before the first cleavage along PC1, while starting to diverge from the earlier stages along PC2. We also quantified the contribution of each tRNA isodecoder to the two principal components using the loading scores (Supplementary Fig. 2b). Consistent with the PCA results, differential expression analysis of nuclear-encoded tRNAs

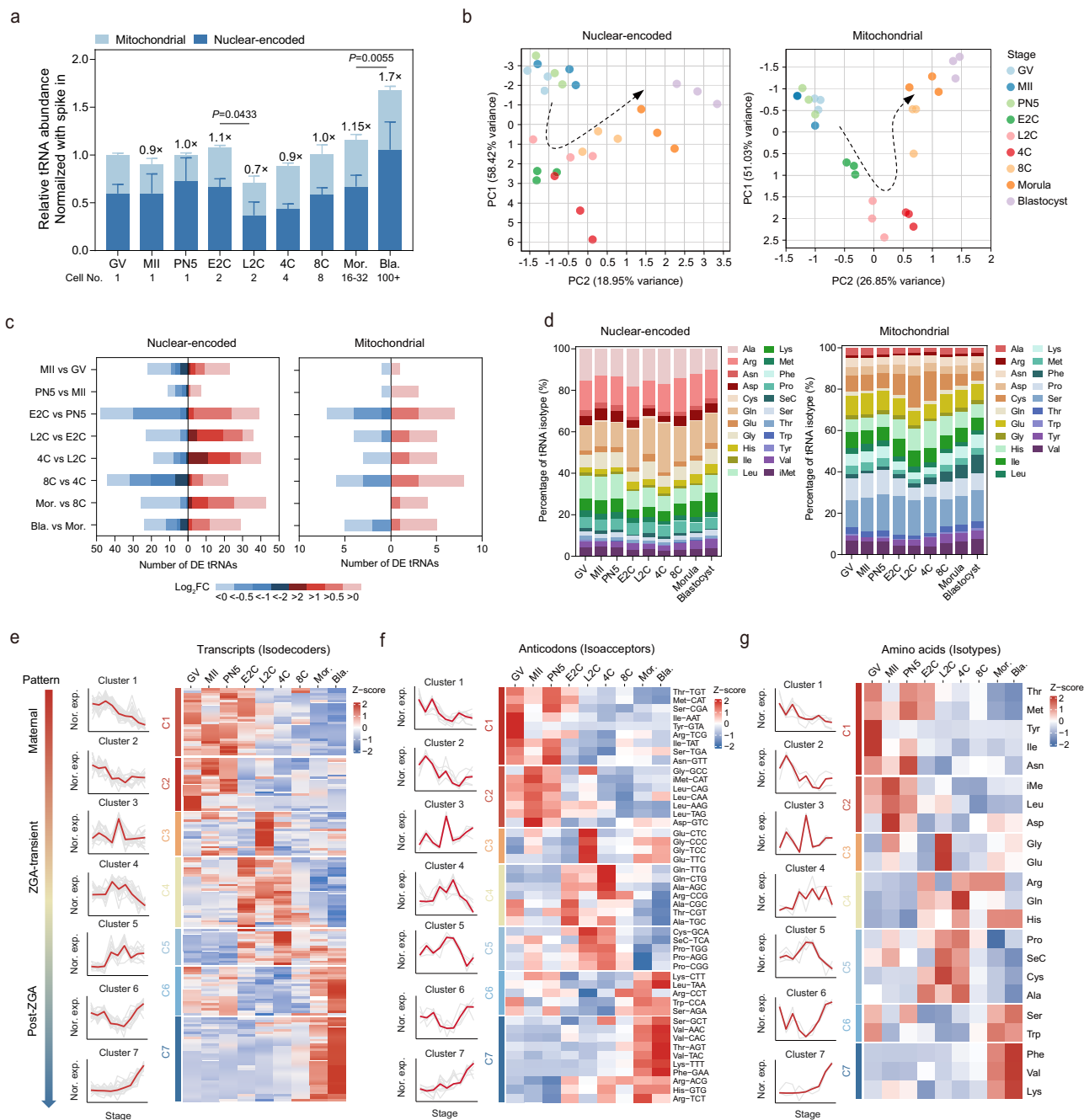


Fig. 2 | Dynamics of mature tRNA pools. a Total abundance of whole embryo tRNAs normalized by Spike-in. Nuclear-encoded, nuclear-encoded tRNA transcripts; Mitochondrial, mitochondrial tRNA transcripts. Bars and error bars represent mean $\pm$ SD ($n = 3$ biological replicates). One-way ANOVA. **b** PCA of all ORACLE-tRNAseq embryo samples. Nuclear-encoded, nuclear-encoded tRNA transcripts; Mitochondrial, mitochondrial tRNA transcripts. PC1, principal component 1, PC2, principal component 2. **c** Number of differentially expressed ($P < 0.05$) tRNA transcripts between adjacent two stages, classified by $\log_2$ FC. **d** The proportion of

tRNA isotypes carrying different amino acids relative to the total tRNA reads. Nuclear-encoded, nuclear-encoded tRNA isotypes. Mitochondrial, mitochondrial tRNA transcripts. **e** K-means based clustering heatmap of tRNA transcript (isocodons) expression and line plots for each cluster, Z-score normalized by row. **f, g** K-means based clustering heatmap of tRNA anticodon (isoacceptor) and tRNA amino acid level (isotype) expression and line plots for each cluster, Z-score normalized by row. **a, c, d** Source data are provided as a Source Data file.

between adjacent stages (Fig. 2c) showed that the adjacent stages before the first cleavage had fewer differentially expressed nuclear-encoded tRNAs, whereas 39 nuclear-encoded tRNAs were upregulated and 48 were downregulated at the first cleavage, and a certain number of differentially expressed tRNAs were observed in each subsequent stage. The PCA and differential expression analysis of mitochondrial tRNAs also showed similar trends (Fig. 2b, c, Supplementary Fig. 2b).

Among all these tRNAs, the tRNA-Gln, tRNA-Arg and tRNA-Ala were the most enriched nuclear-encoded tRNA isotypes in mouse oocytes and embryos, while the mt-tRNA-Ser was the most abundant mitochondrial tRNA (Fig. 2d). Next, we performed K-means clustering on all nuclear-encoded tRNA transcripts across all stages (Fig. 2e, Supplementary Data 1). The clustering results revealed stage-specific highly expressed tRNA clusters, such as C1–C2, which were more highly expressed

before major ZGA, while C3–C5 were mainly highly expressed at the L2C and 4C stages, and C6 and C7 were highly expressed in the lineage specification post-ZGA stages. Concurrently, we performed K-means clustering at both the tRNA anticodon level and the amino acid level, dividing tRNAs into 7 clusters at both the anticodon and amino acid levels (Fig. 2f, g). Similar to the tRNA transcript level, the anticodon and amino acid levels also significantly distinguished embryos at different developmental stages. To visualize the relationships between the seven clusters across the three levels, we generated a Sankey diagram. Despite some variations, the tRNA isodecoder, isoacceptor and isotype pools generally exhibited similar patterns, particularly within C7 (Supplementary Fig. 2c). K-means clustering analysis of mitochondrial tRNAs also revealed embryonic developmental stage-specific features of mitochondrial tRNA expression (Supplementary Fig. 2d), indicating that the mitochondrial tRNA pool was also ever-changing.

tRNA pseudogenes are actively transcribed in embryos and burst at the 4 C stage

ORACLE-tRNAseq annotates tRNA transcripts based on the confidence scores from a genome-wide tRNAscan-SE scan^{26,27}. In the genome-wide scan, a class of tRNAs was

predicted to be pseudogenes (tRXs, Fig. 3a). To assess whether these tRNA pseudogenes are transcribed in embryos, we examined the tRXs that were detectable at each stage of oocyte and early embryo development. We found that most of these tRXs had sequence features of serine tRNAs and were mainly highly expressed at the 4C stage (Fig. 3b, c). Notably, a large proportion of these tRXs were undetectable in the liver (Fig. 3b). Northern blot results also confirmed their high expression in the germline (Fig. 3d).

Since most of these tRXs exhibit tRNA-Ser features, to exclude the possibility that they are artifacts arising from over-modified or degraded tRNA-Ser, we performed sequence alignment between these tRX-Ser and tRNA-Ser. The results showed that, compared with tRNA-Ser, tRX-Ser contains continuous non-homologous regions, continuous base deletions, and scattered mismatches (Fig. 3e), suggesting that they are unlikely to result from mis-mapping.

Using publicly available ATAC-seq²³ and ChIP-seq²⁴ data from mouse embryos, we examined the chromatin states of these expressed and non-expressed tRXs. The results showed that these expressed tRXs had stronger enrichment of Nucleosome-Free Regions (NFRs) and H3K4me3 signals (Fig. 3f), suggesting that these tRXs may be undergoing zygotic transcription. Concurrently, we examined the scores of these tRXs in tRNAscan-SE. We noticed that there was no significant difference in tRNAscan-SE scores between these tRXs and those that were barely detectable in early embryos (Fig. 3g). Combined with the observation that these tRX reads lack CCA tails (Fig. 3h), this suggests a low likelihood of their involvement in normative translation function. These results indicate that these tRXs may have specific regulatory mechanisms and play non-translational roles during embryonic development, rather than being merely ‘silent pseudogenes’.

Transcription activation of tRNA genes coincides with ZGA at the 2-cell stage

We next asked at which stage zygotic tRNA genes are transcriptionally activated at the global level. To this end, we examined the signals of NFRs, H3K4me3, and H3K27me3 at tRNA gene loci (Fig. 4a–c, Supplementary Data 2). The results showed that NFR and H3K4me3 signals began to appear at the 2C stage, and peaked at the 8C stage, then slightly decreased, indicating that tRNA genes started to become accessible at the 2C stage. Furthermore, there was little enrichment of H3K27me3 signals at tRNA gene loci, suggesting that the transcriptional activation of tRNA genes may not be regulated by H3K27me3. To further clarify the relationship between chromatin states and epigenetic marks with tRNA transcription, we examined the abundance of nascent tRNAs (Fig. 4b, Supplementary Data 3). Consistent with the

epigenetic landscape, nascent tRNA levels were low at the GV and PN5 stages, but began to appear abundantly after the E2C stage, and burst at the 4C stage (Fig. 4c). Concurrently, we found that nascent tRNAs exhibited stage-specific expression patterns (Fig. 4d). When we compared the stage-specific expression patterns of nascent and mature tRNAs, we found a positive correlation between the expression patterns of mature tRNAs that were highly expressed after the 2C stage and their nascent tRNA levels, while those highly expressed before the 2C stage showed a tendency toward negative correlation (Fig. 4e). This indicates that the increase in abundance of the former relies mainly on zygotic synthesis, whereas tRNAs highly expressed before the 2C stage are mainly maintained by maternally provided tRNAs, with less impact from zygotic synthesis. For example, Tyr-GTA exhibited a significant maternal high abundance characteristic, but its chromatin accessibility also showed an open state after the 2C stage, similar to Glu-TTC, which is transiently highly expressed during ZGA, and Lys-TTT, which is highly expressed post-ZGA. However, at the nascent level, Lys-TTT, which was expressed later, indeed showed a more consistent dynamic change with its mature tRNA levels (Fig. 4f).

tRNA anticodon dynamics do not reflect global codon usage differences or decoding rate variation

Next, we asked whether changes in the tRNA anticodon pool during early embryonic development reflect differences in decoding rates or accommodate stage-specific global codon usage. During ribosome elongation, tRNAs enter the A-site (Supplementary Fig. 3a). Under otherwise constant conditions, when tRNA availability was limited, ribosome dwell time at the A-site is prolonged. Therefore, in theory, the inverse of tRNA abundance should be proportional to the A-site dwell time. We used Ribo-seq data from early embryonic development⁴ and calculated A-site dwell time based on P-site positioning. Our data showed no significant correlation between 1/tRNA anticodon abundance and relative A-site codon dwell time (Supplementary Fig. 3b), consistent with previous studies using Ribo-seq datasets generated without tetracycline^{14,28}.

We then examined whether A-site dwell time or global codon usage varies across developmental stages. The coefficient of variation (CV) of A-site codon dwell time ranged from 6.71 to 41.7%, whereas the CV of global codon usage ranged from 1.62 to 15.4% (Supplementary Fig. 3c), indicating that dwell times of some codons are strongly regulated, while global codon usage remains largely stable. We further examined the few codons with the largest CV in A-site dwell time and performed Spearman correlation analysis between their A-site dwell time changes and 1/tRNA anticodon abundance variation. A weak, statistically non-significant positive correlation was observed (Supplementary Fig. 3d). Expanding this analysis to all codons and examining the correlation between global codon usage and tRNA anticodon abundance variation, no consistent or significant positive trends were found (Supplementary Fig. 3e).

These results are in agreement with recent studies in human iPSC differentiation systems and zebrafish embryonic models^{13,14,28}. Therefore, during early mouse embryogenesis, dynamic regulation of the tRNA pool is unlikely to serve as a mechanism to alter decoding rates or to optimize translation according to stage-specific global codon usage demands.

tRNA anticodon pool prefers to coordinate with high-translation-efficiency gene pools, rather than stage specific genes

Next, we asked whether the tRNA pool is better adapted to the translation of a specific gene set. First, we examined the correlation between global codon usage and the tRNA anticodon pool. We calculated the global weighted codon usage at each stage using Ribo-seq data from early embryonic development⁴ and found that the Spearman correlation coefficient weighted by Ribosome-protected Fragments (RPF) was

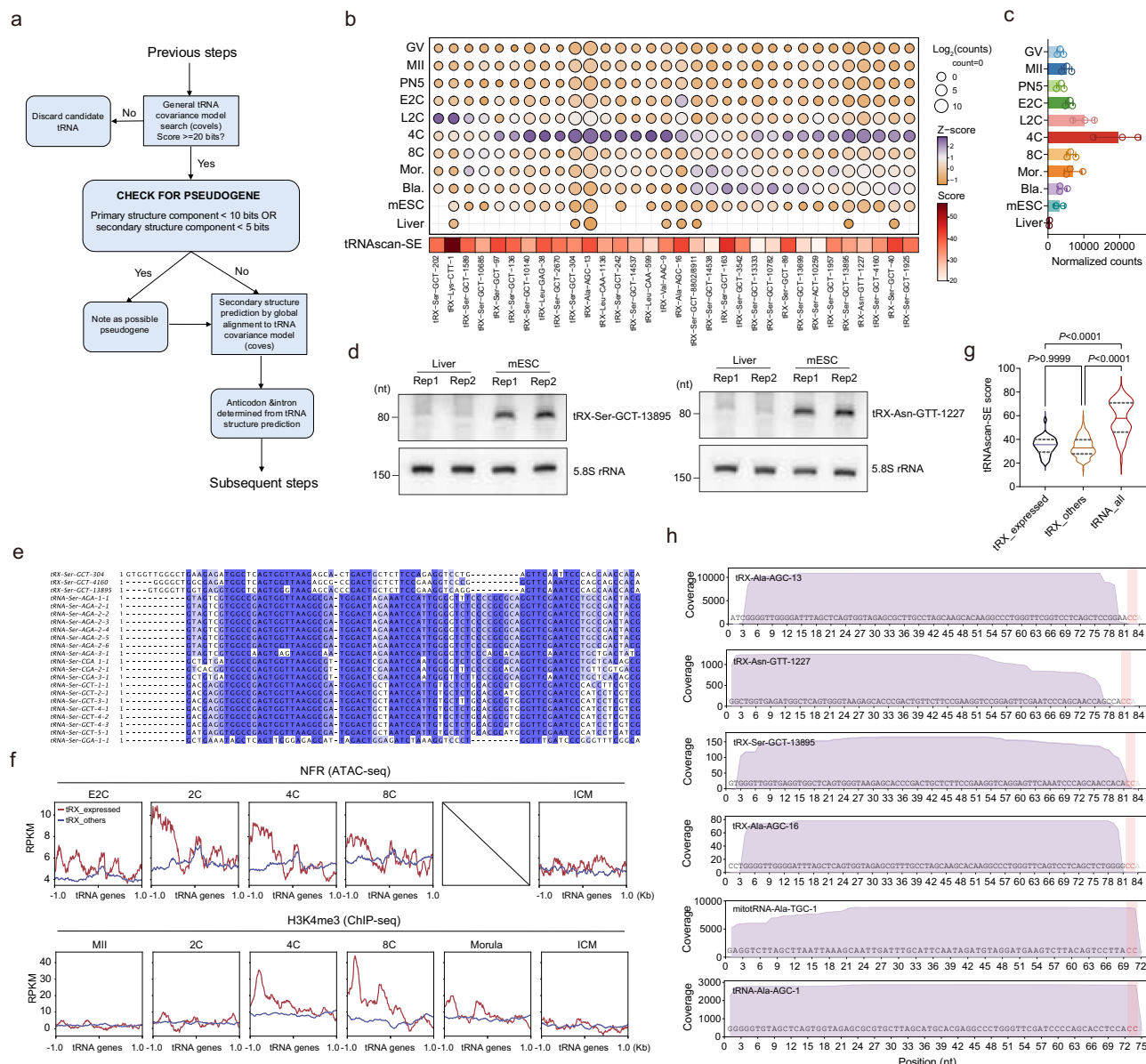


Fig. 3 | Identification of embryo-specifically expressed tRNA pseudogenes.

a Bioinformatic pipeline for the definition of tRNA pseudogenes, redrawn based on Todd M. Lowe et al., *Nucleic Acids Research*, 1997, Fig. 1. **b** Bubble heatmap of tRX abundance changes and corresponding heatmap of tRNA-Scan-SE scores. Bubble, Log₂ expression of tRXs, heatmap Z-score normalized by row. **c** Total abundance changes of identified tRXs across different stages. Scatter plots represent three biological replicates, bars represent the mean, and error bars represent standard deviation. **d** Northern blot validation of tRX abundance differences measured by

ORACLE-tRNAseq. 5.8S rRNA as an internal reference. **e** Multiple sequence alignment of three tRX-Ser and all tRNA-Ser sequences. **f** NFR and H3K4me3 signals at the transcription start site ± 1.0 kb of tRNA genes. **g** Comparison of tRNA-Scan-SE scores among tRXs expressed in embryos, other tRXs, and all predicted tRNAs. Solid line represents the median, dashed line represents the quartiles. Kruskal-Wallis test. **h** Coverage plot showing CCA tail representation of tRXs. **c, d, g** Source data are provided as a Source Data file.

greater than that weighted by mRNA (Fig. 5a), indicating that tRNA pools are better adapted for cellular translation state. Furthermore, the analyses of Spearman correlation between global weighted codon usage and tRNA anticodon proportion at each stage showed a variation range from 0.2134 to 0.3559 ($P = 0.098\text{--}0.0049$) (Fig. 5b), suggesting that the tRNA pool at each stage can globally match the codon usage requirements of the corresponding stage to a certain extent, and the degree of matching varies across stages. Next, we identified seven clusters of stage-specific high-TE genes based on translation efficiency (TE; Supplementary Fig. 4a). These seven clusters of genes exhibited higher TE at a specific embryonic developmental stage (or two, such as MII and PN5). And GO analysis showed that these genes have different functions at their respective stages (Supplementary Fig. 4b). We

hypothesized that one of the reasons for the increased translation efficiency of these genes at specific stages might be better matching of tRNA anticodon pools. However, the Spearman correlation with tRNA anticodon proportion at each stage was lower than the correlation between the global codon usage and tRNA anticodon at the corresponding stage (Fig. 5c), suggesting that the tRNA pool is not specifically optimized for stage-specific mRNA translation. We then wondered whether the tRNA anticodon pool at each stage was better matched to the codon composition of stage high-TE genes, as these genes might have better tRNA anticodon matching given their higher TE. To investigate this, we ranked all genes at each stage by their TE in descending order and selected the top n high-TE gene pools to calculate the weighted codon usage and its correlation with tRNA

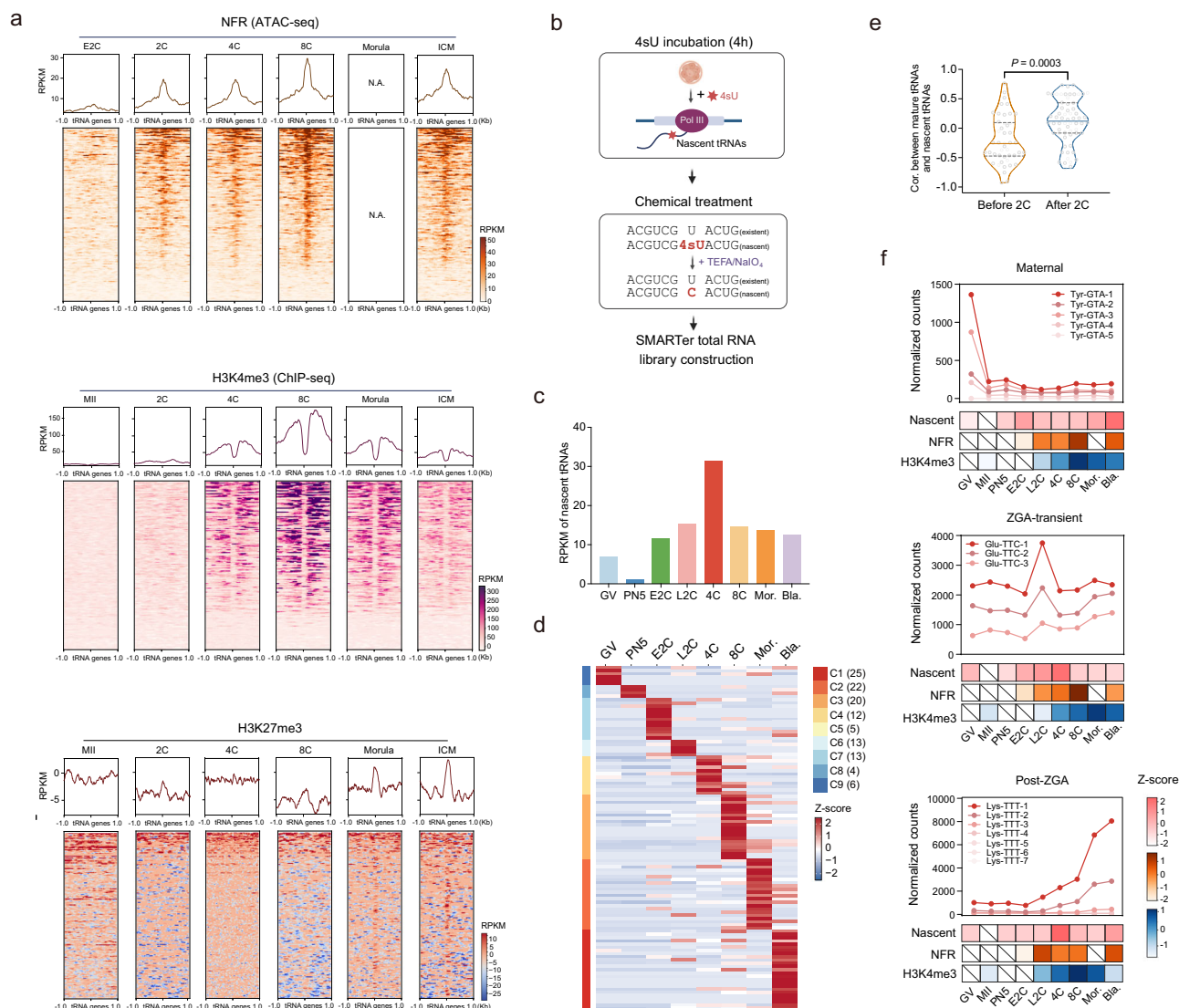


Fig. 4 | Chromatin accessibility and histone modifications of tRNA genes, and nascent tRNA analysis. a NFR, H3K4me3, and H3K27me3 signals at the transcription start site ± 1.0 kb of tRNA genes. N. A., Not Applicable. **b** Method for nascent RNA labeling and chemical conversion workflow, created in BioRender. Li, Y. (2026) <https://BioRender.com/chad1h>. **c** Abundance changes of nascent tRNAs (relative to Total RNA reads). **d** hclust-based clustering heatmap of nascent tRNAs,

Z-Score normalized by row. **e** Stage expression correlation of mature tRNA clusters relatively high abundant before 2C and mature tRNA clusters relatively high abundant after 2C with their corresponding nascent tRNA clusters. **f** Mature tRNA changes of isodecoders corresponding to Tyr-GTA, Glu-TTC, Lys-TTT and changes in corresponding nascent tRNAs, NFR and H3K4me3. Heatmap Z-Score normalized by row. **c**, **e**, **f** Source data are provided as a Source Data file.

anticodon pools (Fig. 5d). We observed that this correlation peaked at a maximum $r = 0.519$ at the 8C stage. As n continued to increase, the correlation gradually decreased and stabilized, eventually reflecting the coordination between the weighted codon usage of global genes and tRNA anticodon pools (Fig. 5e). However, this pattern disappeared after random shuffling of the gene order (Fig. 5d), indicating that the composition of the tRNA anticodon pools is indeed more matched to the codon composition of the high-TE gene pools.

tRNA anticodon pool fine-tunes the translation of post-transcriptional regulators after major ZGA

We observed that after major ZGA, the coordination between the high-TE gene pools and tRNA anticodon pool was significantly increased, while only a slight statistically not significant increase could be observed for the global gene pool (Fig. 6a), indicating a more coordinated relationship between specific mRNA pools and tRNA anticodon supply in translational regulation after major ZGA. To this end,

we analyzed the weighted codon usage of the high-TE gene pools at each stage and compared it with the global weighted codon usage. As expected, the high-TE gene pools showed different codon usage frequencies compared to the global gene pools, and the magnitude of these codon changes was remarkably increased in the stages after major ZGA (Fig. 6b, c). We selected 20 codons (the top 10 with the largest increases and decreases in usage in high-TE gene pools relative to the global gene pool) to evaluate their correlation with anticodon supply at each stage. The results showed that in high-TE gene pools, these 20 key codons exhibited a higher coordination relationship with their corresponding anticodon supply compared to all codons, reaching a correlation score of 0.6426 at the 8C stage (Fig. 6d, e). Notably, the correlation of the usage of these 20 key codons in the global gene pool with tRNA anticodon supply at the 8C stage was slightly worsened compared to all codons (0.2716 vs 0.3502), while the changes were less pronounced at the MII stage (Fig. 6d), indicating that the tRNA anticodon pools indeed preferentially fulfill the usage

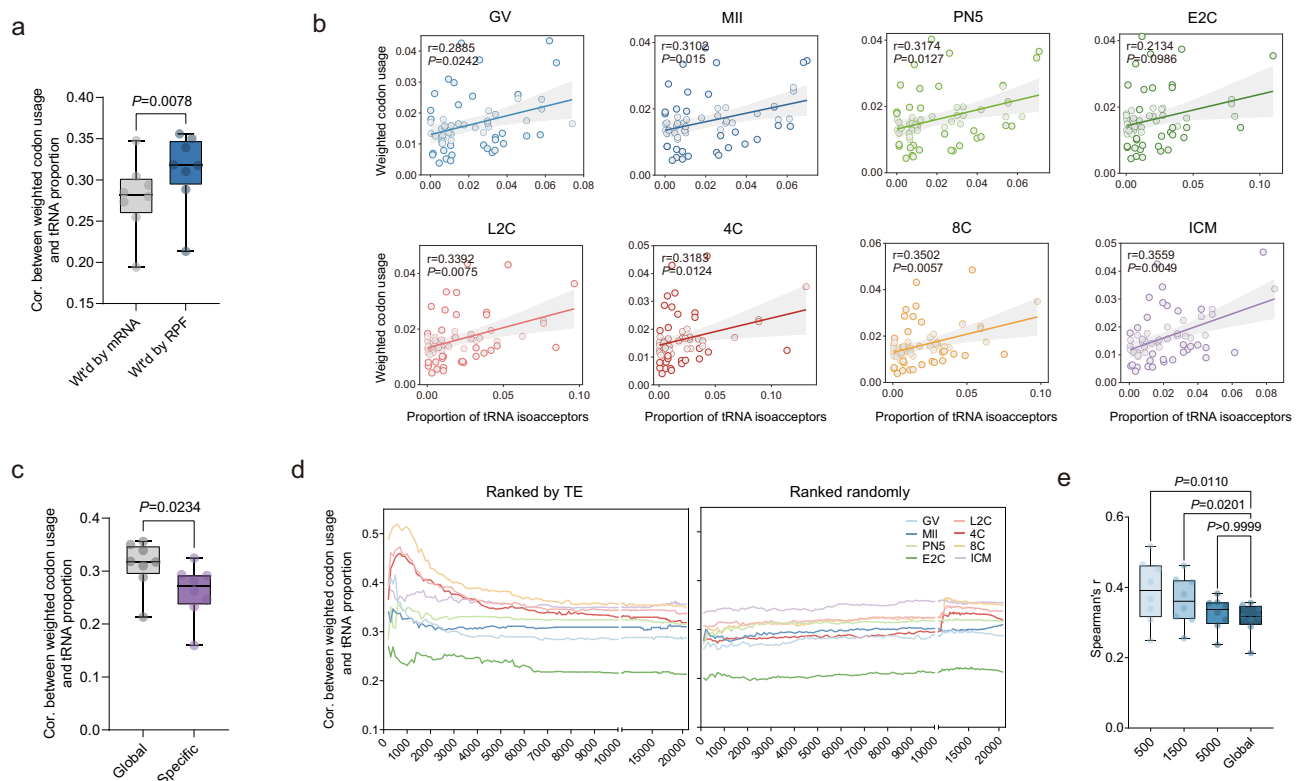


Fig. 5 | Translation coordination between the tRNA anticodon pools and high-TE gene pools. a Box plots of Spearman correlation between global codon usage frequency weighted by mRNA expression and RPF and the proportion of the tRNA anticodon pools. Each point represents a single developmental stage ($n = 8$). Wt'd, Weighted. Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box is the median. Whiskers extend to the minimum and maximum values of the dataset, Wilcoxon paired signed-rank test (two-sided). **b** Scatter plot of Spearman correlation between tRNA anticodon proportion at each stage and global gene weighted codon usage. Fitted line, linear regression, gray shading, 95% confidence interval. P values were calculated using a two-sided Spearman correlation test. **c** Box plots of Spearman correlation between global weighted codon usage frequency and stage-specific high TE gene pools. Each point represents a single developmental stage ($n = 8$). Boxes contain the middle

50% of the data (lower quartile to upper quartile), the horizontal line inside the box is the median. Whiskers extend to the minimum and maximum values of the dataset, Wilcoxon paired signed-rank test (two-sided). **d** Line plots of Spearman correlation between weighted codon usage of the top n gene pools (sorted by TE in descending order or randomized) and the proportion of the tRNA anticodon pools. The Random group's line indicates the median value derived from 50 random iterations. **e** Box plots of Spearman correlation between weighted codon usage frequency of the top 500, 1500, 5000, and all genes (sorted by TE in descending order) and the proportion of the tRNA anticodon pools. Each point represents a single developmental stage ($n = 8$). Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box is the median. Whiskers extend to the minimum and maximum values of the dataset, Wilcoxon paired signed-rank test. **a–e** Source data are provided as a Source Data file.

demand of these key codons in high-TE gene pools after major ZGA. We also examined whether changes in codon usage of the high-TE gene pools before and after major ZGA correlate with changes in the tRNA anticodon pool and obtained negative results (Fig. 6f).

Finally, we asked about the functions of these high-TE gene pools. Interestingly, these genes were mainly enriched in biological terms related to ribosomes, translation, and mRNA splicing and processing (Supplementary Fig. 5a). Transcripts involved in ribosome biogenesis and mRNA processing are typically highly abundant, as they represent housekeeping functions required for protein synthesis in all cells. To exclude background effects introduced by ranking based on TE, we first analyzed the correlation among TE, RPF, and RNA read counts. The results showed that the correlation between TE and RPF or RNA levels was moderate and much lower than that between RPF and RNA (Fig. 6g–h), indicating that high-TE genes are not necessarily highly expressed. We further examined gene sets that account for 50% or 80% of total mRNA reads at each stage and compared them with the high-TE gene pools identified here. The results showed that these high-TE pools are not dominated by highly expressed genes; for example, at the 8 C stage, the high-TE pool of 600 genes contained only 10 genes from the 50% mRNA set and 69 genes from the 80% mRNA set (Fig. 6i–j). We also sorted the high-TE gene pools by mRNA abundance

and divided them into two groups: above-average and below-average expression, then performed GO analysis separately. Both groups clustered into ribosomal proteins and mRNA processing factors (Supplementary Fig. 5b). In contrast, other frequently observed GO terms, such as mitotic cell cycle, DNA replication, and glycolytic process, were relatively underrepresented in these gene sets (Fig. 6k). In summary, we excluded background effects and validated the reliability of the GO enrichment results. After major ZGA, the transcription and translation of the zygotic genome are activated. Therefore, the enhanced translational coordination between the tRNA anticodon pools and high-TE gene pools may favor the preferential translation of these mRNAs (such as ribosomal mRNAs) to ensure the supply of housekeeping proteins (such as ribosomal proteins) during the transcriptionally and translationally active period, thereby guaranteeing overall translational demands (Fig. 6j).

ORACLE-tRNAseq reveals dynamic signatures of tRNA modifications

Modifications at the Watson-Crick face are prevalent in tRNAs, including m¹G, m¹A, m³C and others²⁹, and these impede reverse transcription and hinder accurate sequencing. AlkB can be used in tRNA library preparation to remove several key Watson-Crick face

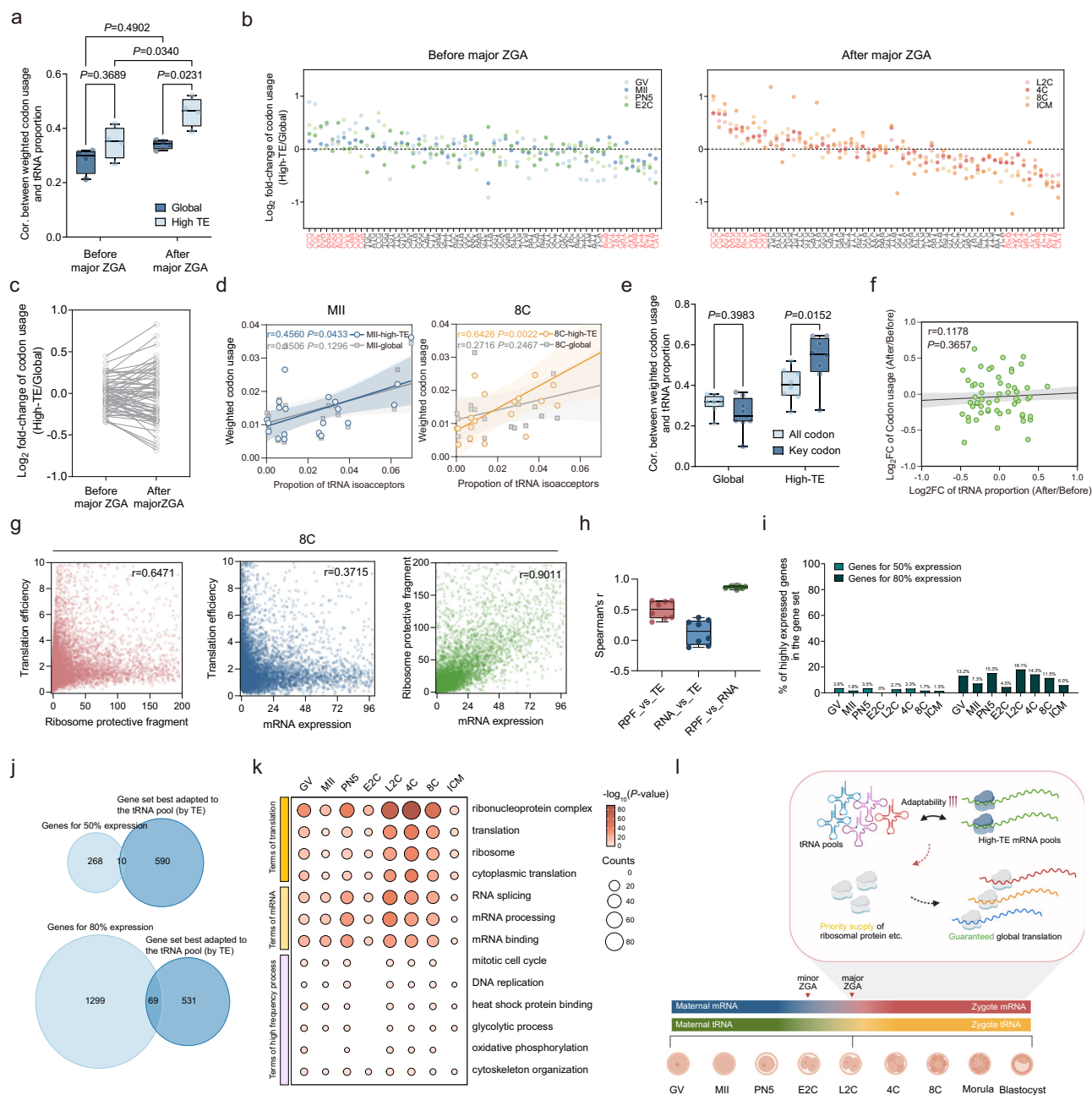


Fig. 6 | Alteration of translation coordination after major ZGA. a Box plots of Spearman correlation between weighted codon usage of global genes and high-TE gene pools before and after major ZGA and the proportion of the tRNA anticodon pools. Each point represents a single developmental stage ($n = 4$). Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box is the median. Whiskers extend to the minimum and maximum values of the dataset, Two-way ANOVA. P values were calculated using a two-sided Spearman correlation test. **b** Log₂ FC of weighted codon usage of high-TE gene pools compared to global genes before and after major ZGA. Each point represents one codon ($n = 61$). **c** Changes in Log₂ FC of weighted codon usage of high-TE gene pools compared to global genes before and after major ZGA. Each point represents one codon ($n = 61$). **d** Spearman correlation between weighted codon usage frequency of global genes and high-TE gene pools for the 20 key codons and the proportion of corresponding tRNA anticodon pools at MII or 8C stage. Fitted line, linear regression, shading, 95% confidence interval. P values were calculated using a two-sided Spearman correlation test. **e** Spearman correlation between weighted codon usage frequency of global genes and high-TE gene pools for the 20 key codons and the proportion of corresponding tRNA anticodon pools across all stages. Each point represents a single developmental stage ($n = 8$). Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box indicates the median, and whiskers extend to the minimum and maximum values. P values were calculated using a two-sided Spearman correlation test. **f** Spearman correlation plot between the Log₂FC of weighted codon usage in the high-TE gene pool and the Log₂FC of tRNA anticodon pool proportion before and after ZGA. Fitted line, linear regression, shading, 95% confidence interval. **g** Scatter plots showing the correlations among TE, RPF, and RNA. Correlations were calculated using Spearman's r . **h** Boxplots of correlations among TE, RPF, and RNA across all eight developmental stages. Each point represents a single developmental stage ($n = 8$). Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box indicates the median, and whiskers extend to the minimum and maximum values. **i** Percentage of genes contributing to 50% or 80% of expression within the gene set best adapted to the tRNA pool across all eight stages. P values were calculated using a two-sided Spearman correlation test. **j** Venn diagram showing the overlap between the gene set best adapted to the tRNA pool at the 8-cell stage and the genes contributing to 50% or 80% of expression at the same stage. **k** GO enrichment analysis results for the high-TE gene sets identified at each stage. **l** Schematics of translational coordination between the tRNA pool and the high-TE gene pools. Created in BioRender. Li, Y. (2026) <https://BioRender.com/cli2nna>. **a–k** Source data are provided as a Source Data file.

and whiskers extend to the minimum and maximum values. P values were calculated using a two-sided Spearman correlation test. **f** Spearman correlation plot between the Log₂FC of weighted codon usage in the high-TE gene pool and the Log₂FC of tRNA anticodon pool proportion before and after ZGA. Fitted line, linear regression, shading, 95% confidence interval. **g** Scatter plots showing the correlations among TE, RPF, and RNA. Correlations were calculated using Spearman's r . **h** Boxplots of correlations among TE, RPF, and RNA across all eight developmental stages. Each point represents a single developmental stage ($n = 8$). Boxes contain the middle 50% of the data (lower quartile to upper quartile), the horizontal line inside the box indicates the median, and whiskers extend to the minimum and maximum values. **i** Percentage of genes contributing to 50% or 80% of expression within the gene set best adapted to the tRNA pool across all eight stages. P values were calculated using a two-sided Spearman correlation test. **j** Venn diagram showing the overlap between the gene set best adapted to the tRNA pool at the 8-cell stage and the genes contributing to 50% or 80% of expression at the same stage. **k** GO enrichment analysis results for the high-TE gene sets identified at each stage. **l** Schematics of translational coordination between the tRNA pool and the high-TE gene pools. Created in BioRender. Li, Y. (2026) <https://BioRender.com/cli2nna>. **a–k** Source data are provided as a Source Data file.

modifications^{15,16}. However, it is difficult to recover minute amounts of RNA from the AlkB reaction buffer, rendering this treatment unusable. However, several reverse transcriptases, such as TGIRT, have been previously reported to improve read-through during cDNA synthesis³⁰, and these can achieve satisfactory results in AlkB-free protocols¹⁷.

ORACLE-tRNAseq utilizes the Diagenode® RNA reverse transcriptase for cDNA synthesis, and we observed that the Diagenode® RNA reverse transcriptase caused high levels of misincorporation at several AlkB-demodified sites on tRNAs, such as m¹G at position 9, m²G at position 26, m³C at position 32, and m¹A at position 58, indicating that it has great read-through capability at specific modification sites, and the proportion of misincorporation at these sites was significantly reduced in tRNA libraries treated with AlkB (Supplementary Fig. 6a). A small-scale spike-in benchmark also demonstrated that ORACLE-tRNAseq provides absolute quantification for specific modifications (Supplementary Fig. 6b), which enabled the validation and downstream analysis of RNA modifications on tRNAs by monitoring changes in misincorporation at specific sites.

Besides several AlkB-sensitive RNA modification sites, other sites such as Position 20/20a(U), Position 34(A), and Position 37(G) also caused misincorporation, we annotated these positions with reference to the existing literature^{31–33} (Fig. 7a). We quantified the misincorporation levels at all these sites and found that, apart from Position 34(A), which is stably and highly modified across all stages, many sites showed dynamic changes in misincorporation during ZGA (Fig. 7b, Supplementary Fig. 6c). Notably, Position 58(A), annotated with m¹A, which is important for tRNA maturation and stability, displayed two distinct patterns: For observed modification rate, one class of tRNA transcripts decreased after the first cleavage and recovered after the 4-cell stage, while the other class started to decrease after L2C and slightly increased until the blastocyst stage (Fig. 7c, Supplementary Data 4).

We employed LC-MS/MS to quantify 19 types of detectable RNA modifications at the global RNA level across oocyte and early embryonic development (Supplementary Fig. 7a–c, Supplementary Data 5). Although these bulk-level measurements may reflect both changes in RNA composition and genuine shifts in modification states, they revealed pronounced stage-dependent differences. Several modifications were enriched before the first cleavage, others accumulated after cleavage and declined during lineage specification, while a subset peaked transiently at the morula stage. Overall, modification levels were lowest at the blastocyst stage. Notably, while ORACLE-tRNAseq revealed relatively stable m²G26 levels across development, bulk LC-MS/MS analysis showed an apparent increase at the morula stage. This discrepancy likely reflects differences in measurement principles, as LC-MS/MS quantifies total nucleoside abundance whereas ORACLE-tRNAseq measures site-specific modification occupancy. The transient rise in m²G may thus result from changes in RNA composition, such as shifts in RNA species distribution, rather than altered modification rates.

Increasing evidence suggests that modifications on tRNAs are interdependent and interact with each other³⁴. Using Single-read Analysis of Crosstalks (SLAC), an analytical pipeline for tRNA crosstalk previously developed³⁵, we were able to directly analyze and visualize these tRNA crosstalks in our ORACLE-tRNAseq data. We identified two notable examples: one is Asp-GTC-1, where the odds ratio value for Positions 6 (G) and 9 (A) significantly increased at L2C, indicating an elevated level of co-occurrence of modifications at these two sites, and the other is Arg-TCG-3, where the mutual exclusivity of modifications at Positions 7 (G) and 9 (G) increased after the 4-cell stage (Fig. 7d). While Position 9(A) or Position 9(G) was identified as m¹A and m¹G sites, the modification status of Position 6(G) and Position 7(G) remained unclear. Therefore, we analyzed the misincorporation patterns at classical modification sites (Supplementary Fig. 8a) and found that when the upstream sequence is C, only Position 26 being G

generates misincorporation. This site is typically annotated as m²G. We compared the misincorporation patterns with those at the two sites of interest. The results showed that Asp-GTC-1 position 6 displays a mixture of C and T mismatches, which resembles the mismatch pattern observed at Ala-AGC-1 position 26. Arg-TCG-3 position 7 shows predominantly A mismatches, similar to Ser-TCG-2 position 26, but with a stronger bias toward a single mismatch type (Fig. 7e). To address whether these mismatches could result from Single Nucleotide Polymorphisms (SNPs), we performed PCR and sequencing of the corresponding tRNA gene loci from genomic DNA of the C57BL/6N mouse strain. All copies of Arg-TCG-3 (two gene copies) and Asp-GTC-1 (thirteen gene copies) showed single peaks at the respective positions (G at position 7/6), indicating no SNPs or sequence heterogeneity among copies (Supplementary Fig. 8b). These observations provide suggestive evidence for the potential occurrence of modifications and crosstalk at these specific sites. Taken together, these results highlight the complex crosstalk and fine-tuned regulation of tRNA modifications during early embryonic development.

Discussion

Studying tRNAomes in scarce samples like mammalian embryos has been challenging. ORACLE-tRNAseq provides a solution by enabling tRNA library generation from as few as five embryos through its ligation-free, single-tube reaction coupled with UMI-based PCR duplicate removal, contrasting with existing methods that require higher RNA input. While other tRNA sequencing methods often involve more complex procedures to address specific needs—such as detecting tRNA aminoacylation or enhancing capture efficiency of tRNA molecules—as well as adopting approaches that retain all reverse transcription fragments to reduce bias, ORACLE-tRNAseq significantly broadens the scope of tRNA research. Although we have not specifically evaluated how template-switching preferences of structured tRNAs and RT behavior might influence quantification, it is worth emphasizing that ORACLE-tRNAseq still demonstrates quantitative consistency with other bulk tRNA-seq methods. By overcoming the limitations of sample availability, it makes the study of tRNAs in precious materials such as mammalian early embryos, as well as other contexts with limited cell numbers, such as profiling tRNA pools in immune and hematopoietic stem cell differentiation and comparative studies of embryonic tRNAomes across species, a practical reality. This advancement thereby opens new avenues for investigating tRNA roles in diverse biological processes.

Significantly distinct maternal and zygotic tRNA pools have been previously observed in zebrafish embryos^{33,34}. Our analysis of the expression of mouse embryonic tRNA pools revealed a clear distinction between maternal and zygotic stages, with this distinction emerging after the first cleavage and continuing to evolve until the blastocyst stage. Furthermore, our investigation of tRNA gene chromatin accessibility and epigenetic landscape showed that these tRNA genes become more accessible and transcriptionally active from the 2-cell stage, coinciding with ZGA. A recent study also demonstrated that the global level of 5fC increases during *Xenopus* ZGA and accumulates extensively at active tRNA tandem repeats, leading to the recruitment of Pol III and promoting tRNA transcription³⁶. This is also consistent with our observation of a large amount of nascent tRNAs appearing at the onset of ZGA.

Generally, tRNA pseudogenes are considered tRNA-like sequences that may lack transcriptional or functional activity²⁷. However, as many tRNA pseudogenes still retain promoter sequences internally, they may be transcribed at low levels by RNA polymerase III. Despite being long regarded as non-functional genomic redundancy, there is still sparse evidence suggesting that tRNA pseudogenes may produce RNA molecules with potential regulatory functions through low-level transcription, for example, potentially affecting normal tRNA metabolism by competing for common factors or being misprocessed³⁷.

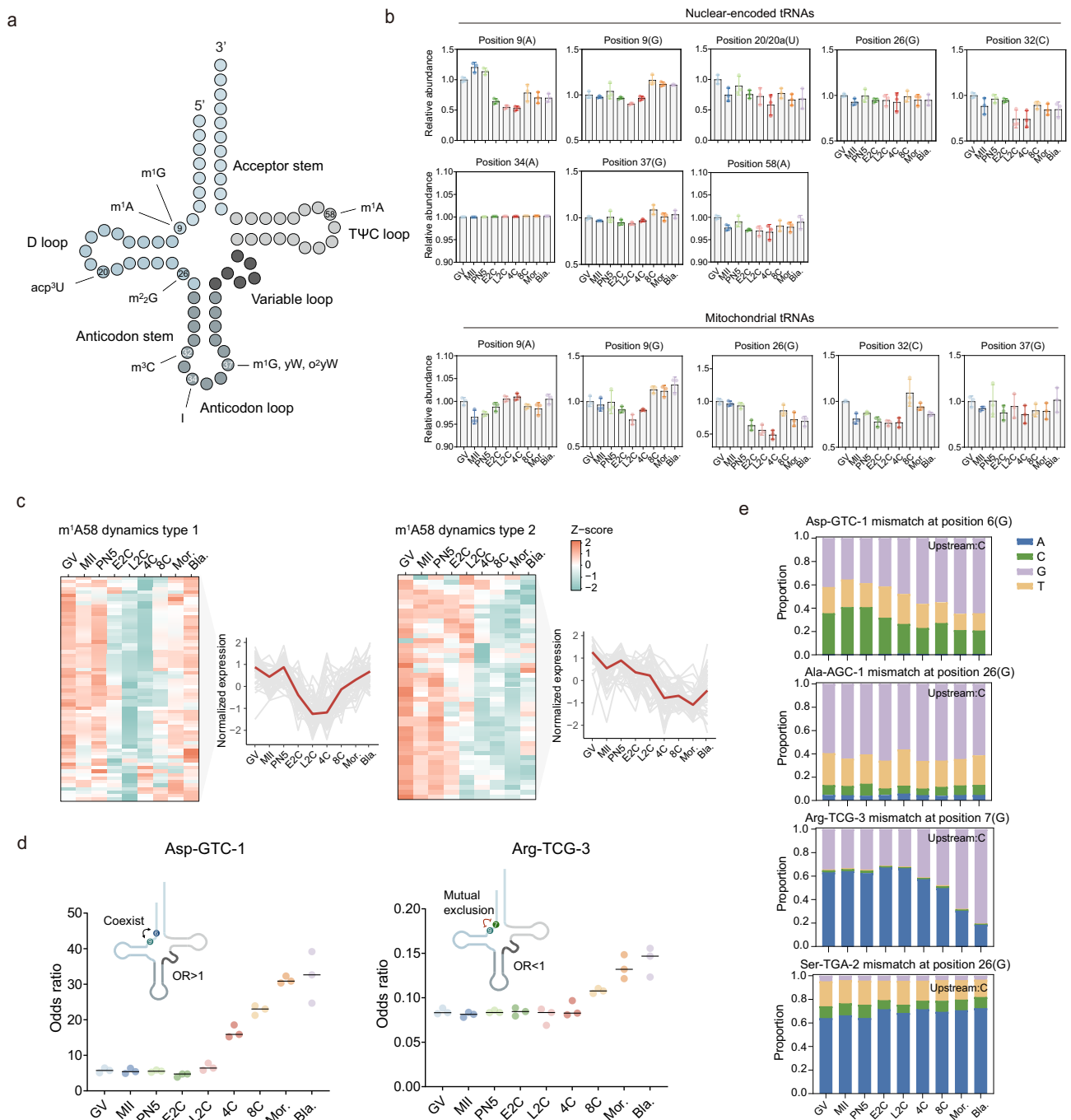


Fig. 7 | tRNA modifications analysis using ORACLE-tRNAseq. a A tRNA model showing the types of modifications present at typical misincorporation sites. **b** Relative changes in modification frequencies of eight modification sites on nuclear-encoded tRNAs or Mitochondrial tRNAs, normalized to the modification frequency in GV oocytes. Scatter plots represent three biological replicates, bars represent the mean, and error bars represent standard deviation. **c** K-means based

clustering heatmap of m¹A₅₈ modification changes across different tRNA transcripts and line plots for the two clusters. **d** Crosstalk of RNA modifications at positions 6 and 9 of Asp-GTC-1 and at positions 7 and 9 of Arg-TCG-3. Center line indicates the median. **e** Stacked bar plots showing changes in the proportion of mismatches at specific nucleotide positions of selected tRNA isodecoders across developmental stages. **b**, **d**, **e** Source data are provided as a Source Data file.

Furthermore, tRNA pseudogenes may acquire new functions during evolution. For instance, some specific tRNA-Asp transcripts with poorly defined higher-order structures can act as molecular decoys, interfering with RNA interactions or affecting mRNA stability³⁸, while the tRNA-derived Short Interspersed Nuclear Elements (SINEs) genes BC1 and BC200 regulate the synthesis of synaptic proteins in the brain³⁹. Our observation of upregulated tRNA pseudogenes in mouse embryos at the 4-cell stage and their silencing in differentiated tissues

such as the liver further highlights the complexity of tRNA pseudogene regulation and suggests their potential non-canonical functions in early development, which warrant further investigation.

Our study explored the functional significance of the embryonic tRNA pool by integrating tRNA-seq data with existing ribosome profiling and mRNA-seq datasets. We found that the tRNA pool is generally better adapted for the translation of high-TE gene pools rather than gene pools with stage-specific high translation efficiency. Consistent

with previous conclusions^{13,14,28}, we did not observe changes in the tRNA pool that reflect variations in ribosome A-site decoding rates or adaptations to global codon usage. Notably, the adaptation of the tRNA pools to high-TE gene pools significantly increased after major ZGA, suggesting a shift in translational coordination that may prioritize the synthesis of essential housekeeping proteins, such as ribosomal components and mRNA processing factors, during the rapid transcriptional and translational activation following ZGA. The more pronounced codon usage bias in high-TE gene pools after major ZGA and the better match with available tRNA anticodons further support this optimized resource allocation. Thus, our findings propose a model where the embryo exhibits translational coordination between the tRNA pool and high-TE gene pools after zygotic genome activation, potentially ensuring efficient production of housekeeping proteins necessary to support increased transcriptional and translational demands during this critical developmental window.

Methods

Animals

All animal experiments and husbandry practices were conducted in accordance with institutional guidelines and approved by the Tongji University Animal Care and Use Committee (Approval No. TJAB07623101). Six weeks old female C57BL/6N mouse and 10 weeks old male mouse were given access to food and water ad libitum and were maintained on a 12:12 h light-dark artificial lighting cycle. Mouse were housed in cages at a temperature of 22–25 °C, with 40–60% humidity.

Cell culture

The 46 C mESCs used in this study were kindly provided by Dr. Qilong Ying (University of Southern California, USA) and were cultured in DMEM medium (10569010, Gibco) supplemented with 20% FBS (Gibco, 30044333), 1% MEM Non-Essential Amino Acids Solution (Gibco, 11140050), 1% Penicillin/Streptomycin (BasalMedia, S110JV), 1% EmbryoMax Nucleoside (Sigma-Aldrich, ES-008-D), 100 μ M β -mercaptoethanol (31350-010; Thermo Fisher Scientific), 0.1% mouse leukemia inhibitory factor (Sigma-Aldrich, ESG1106), 1 μ M PD0325901 (Selleck, S1036), and 3 μ M CHIR99021 (Selleck, S2924) on gelatin-coated adherent cell culture dishes at 37 °C with 5% CO₂. Passage 0 (P0) MEFs were derived from C57BL/6N mouse embryos and were kindly provided by Dr. Zhili Deng (Xiangya Hospital, China). MEF cells and HEK-293T cells were cultured in DMEM medium supplemented with 10% FBS (Gibco, A5256701), 1% MEM Non-Essential Amino Acids Solution, and 1% Penicillin/Streptomycin at 37 °C with 5% CO₂.

Total RNA extraction

For cultured cells, cells were washed with DPBS and directly lysed with 1 mL TRIzol (Invitrogen, 15596018). Cells were scraped off the dish and homogenized by pipetting. For liver tissue, C57BL/6N mice were euthanized, and the liver was rapidly frozen in liquid nitrogen. Approximately 1 mg of tissue was cut, ground, and homogenized in 1 mL TRIzol by pipetting. One-fifth volume of 1-Bromo-3-chloropropane (Sigma-Aldrich, B9673) was added to the TRIzol-containing samples, and the mixture was vortexed. After a brief incubation, the samples were centrifuged at 10,000 $\times g$ for 5 min at 4 °C. The supernatant was collected, and 2.5 volumes of anhydrous ethanol and 1 μ L of linear acrylamide (Invitrogen, AM9520) were added, followed by incubation at –20 °C for 30 min. The samples were then centrifuged at 16,000 $\times g$ for 30 min at 4 °C, and the pellet was retained. The pellet was washed with 1 mL of 75% ethanol, briefly centrifuged at 10,000 $\times g$ for 5 min, and the supernatant was completely removed. The resulting pellet was dissolved in ultrapure water (Invitrogen, 10977015), and the RNA concentration was measured using a Nanodrop One (Thermo Fisher Scientific, USA) or Qubit 4 (Thermo Fisher Scientific, USA) and stored at –80 °C.

T4PNK treatment

Extracted total RNA (1 μ g) was treated with 10 U of T4 Polynucleotide Kinase (New England Biolabs, M0201) in 100 μ L of reaction mixture containing 1 $\times$ T4 Polynucleotide Kinase Reaction Buffer and 1 mM ATP at 37 °C for 20 min. Subsequently, three volumes of anhydrous ethanol and 1 μ L of linear acrylamide were added, and the mixture was incubated at –20 °C for 30 min. The samples were centrifuged at 16,000 $\times g$ for 30 min at 4 °C, and the pellet was retained and washed with 75% ethanol. The resulting pellet was dissolved in ultrapure water, and the RNA concentration was measured using a Nanodrop One or Qubit 4 and stored at –80 °C.

AlkB treatment

Extracted total RNA (1 μ g) was treated in a 100 μ L reaction mixture containing 50 mM HEPES (pH 8.0, Sigma-Aldrich, H3375), 75 mM ammonium iron(II) sulfate (pH 5.0, Sigma-Aldrich, FX0245), 1 mM α -ketoglutaric acid (Sigma-Aldrich, K1128), 2 mM sodium ascorbate (Sigma-Aldrich, 1613509), 1 μ g AlkB (produced in our lab), and 5% (vol/vol) RNase Inhibitor (Takara, 2313) at 37 °C for 30 min. Total RNA extraction was then performed by adding three volumes of TRIzol LS (Invitrogen, 10296010) and following the Total RNA extraction procedure described above.

Northern blot

Two micrograms of sample were separated on a 10% Acrylamide/Bisacrylamide (19:1 mix, Ambion, AM9022) gel containing 8 M urea in 1 $\times$ TBE (Invitrogen, AM9863) at 150 V, and the bands were visualized using 1 $\times$ SYBR Gold (Thermo Fisher Scientific, S11494) stain with a Tanon MINI Space Gel Analysis System (Tanon, China). RNA bands in the gel were transferred to a nylon membrane (Sigma-Aldrich, 11417240001) in 1 $\times$ TBE at 100 mA for 1 h, followed by crosslinking using a CL-3000 UV crosslinker (UVP, USA) at 0.12 J/cm² for 12 s. The following 5'-DIG-labeled DNA probes were used to detect specific tRNA species: tRNA-Gly-CCC: 5'-AATTCTACCATTGAACCAC-3'; mitotRNA-Trp-TCA: 5'-GCCGACTAGTATATCCTAA-3'; tRX-Ser-GCT-13895: 5'-GCTCTTACCCACTGAGCCACCTCACCAACC-3'; tRX-Asn-GTT-1227: 5'-GTGCTCTTACCCACTGAGCCATCTCACCAG-3'; 5.8S rRNA: 5'-AGC-GACGCTCAGACAGGCGT-3'. Hybridization was performed in DIG Easy Hyb buffer (Roche, 11796895001) at 42 °C on a horizontal shaker at 50 rpm for 12 h. After removing the hybridization solution, the membrane was washed twice at 42 °C with low stringent buffer (2 $\times$ SSC and 0.1% (wt/vol) SDS) for 10 min each time. This was followed by two washes with high stringent buffer (0.1 $\times$ SSC and 0.1% (wt/vol) SDS) for 10 min each time. Finally, the membrane was washed with washing buffer (1 $\times$ SSC) for 10 min. The membrane was then blocked with Blocking Solution (Roche, 11096176001) at working concentration for 3 h at room temperature. Subsequently, Anti-Digoxigenin-AP Fab fragments (Roche, 11093274910) were added to the blocking solution at a dilution of 1:10,000 and incubated again for 1 h at room temperature. Next, the membrane was washed four times for 15 min each time with Dig-washing buffer (1 $\times$ maleic acid buffer and 0.3% Tween-20) at room temperature. After washing, the membrane was incubated with Dig-detection buffer (0.1 M Tris and 0.1 M NaCl, pH 9.5) for 10 min at room temperature. Finally, 1 $\times$ CSPD (Roche, 11755633001) was overlaid onto the membrane in the dark at 37 °C for 15 min, and the signal was detected using a Tanon 5200 Chemiluminescence Analysis System (Tanon, China).

Embryo collection

C57BL/6N female mice were euthanized, and germinal vesicle (GV) oocytes were collected from the ovaries 48 h after intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG). For collection of MII oocytes, mice were superovulated by intraperitoneal injection of 5 IU PMSG followed by 5 IU human chorionic gonadotropin (hCG) 48 h later, and MII oocytes were collected from the oviducts 16 h

after hCG injection. For collection of early embryo samples, mice were euthanized, and after collecting MII oocytes, sperm were immediately capacitated for 20 min in HIF fertilization medium (Nanjing Aibei Biotechnology, Co.m1135), followed by 6 h of in vitro fertilization. After 6 h of fertilization, excess sperm were removed, and fertilized embryos with a second polar body were selected and transferred to G1-PLUS culture medium (Vitrolife, 512971) for culture at 37 °C with 5% CO₂. PNS embryos were collected at 8 h post-fertilization, early 2-cell (E2C) embryos at 24 h, late 2-cell (L2C) embryos at 36 h, 4-cell (4C) embryos at 48 h, 8-cell (8C) embryos at 60 h, Morula embryos at 72 h, and Blastocyst embryos at 96 h.

ORACLE-tRNAseq library construction

One hundred cells or five embryos were included in 5 µL of a mixture at pH 9.0 containing 5% (vol/vol) RNase Inhibitor, 0.25 mM Tris-HCl (Sigma-Aldrich, 10708976001), 2 pg of spike-in (synthetic E. coli tRNA-Lys-UUU-CCA: 5'-GGGUCGUUAGCUCAGUUGGAGAGCAGUUGA-CUUUUAAUCAAUUGGUCGACAGGUUCGAUCCUGCAGCCACCA-3'), and 1× lysis buffer (Takara, 635013), heated at 72 °C for 2 min, and immediately placed on ice for 5 min. Subsequently, the samples were incubated at 37 °C for 30 min to remove amino acids and then supplemented with ultrapure water to 8 µL. For spike-in benchmark, synthetic tRNA-Arg-CCT-2 with m1G9: 5'-GCCCCA-GUGGCCUAAUGGAUAGGCACUGGCCUUAAGCCAGGGAUUGGUGGUUCGAGUCCACCUUGGGGUGCCA-3' and tRNA-Met-CAT-1 with or w/o m1A58: 5'-GCCUCGUUAGCGCAGUAGGUAGCGCGUACGUCUCAUUAUCUGAAGGUCGUGAGUUCGAUCCUCA-CACGGGGCACCA-3' were mixed at defined molar ratio to a final mass of 400 pg. Library construction was performed using the D-Plex Small RNA-seq Kit for Illumina (Diagenode, C05030001). Briefly, 2 µL of Dephosphorylation Buffer, 5 µL of Crowding Buffer, 1 µL of Small Tailing Buffer, and 0.5 µL of Small Tailing Reagent were added to the 8 µL mixture, followed by polyadenylation at 37 °C for 40 min. One microliter of Reverse Transcription Primer was added, and the mixture was heated at 70 °C for 10 min and cooled to 25 °C at a rate of 0.5 °C/s, holding for 2 min. Five microliters of Reverse Transcription Buffer and 1 µL of Reverse Transcription Reagent were added, and the mixture was incubated at 25 °C for 15 min. Two microliters of STSO were added, and reverse transcription was performed at 42 °C for 2 h, followed by incubation at 70 °C for 10 min. Then, 10 µL of D-Plex Forward Primer, 10 µL of indexed D-Plex Reverse Primer, and 50 µL of PCR Master Mix were added, and PCR was performed using the following program: 98 °C for 30 s, 14–24 cycles of 98 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s, followed by 72 °C for 10 min and holding at 4 °C. Libraries were then cleaned up using AMPure XP beads (Beckman, A63881), and the bands were recovered from an 8% Acrylamide/Bisacrylamide (37.5:1 mix, Bio-red, 1610158) gel. Sequencing was performed on an Illumina Novaseq XPlus sequencer (Illumina, USA).

ORACLE-tRNAseq data processing

Data were processed using *fumitools* (v0.19.0) with default commands and parameters to extract UMIs. Reads were trimmed using *cutadapt* (v4.4, <https://github.com/marcelm/cutadapt/blob/main/doc/guide.rst>) with the following parameters: --match-read-wildcards -u 16 -a 'AAAAAAAAAAAAAAAAAAAA' --trimmed-only, followed by -a 'AAAA\$' -a 'AA\$' -a 'A\$' -m 50 -M 110. The *mim-tRNAseq* pipeline (v1.3) was used to align reads to tRNA sequences with the following parameters: --species Mmus --cluster-id 0.97 --min-cov 0.0005 --max-mis-matches 0.1. The aligned BAM files were deduplicated using *UMI tools*⁴⁰ (v1.1.6) with default commands and parameters and converted back to fastq files. Finally, alignment was performed again using the *mim-tRNAseq* pipeline with the previous parameters. The first 96% of the tRNA sequence was defined as full-length (i.e., excluding CCA) due to the absence of the 3' terminal A. The tRNA transcript matrix was normalized using the *DESeq2* (v1.44.0) package in R, averaging the

three replicates and filtering rows with zero values. K-means clustering and visualization were performed using the *ClusterGVis* (v0.1.1) package in R.

Analysis of other RNA species

The bam files of reads categorized as “No mapping” in the *mim-tRNAseq* pipeline were converted back to fastq files and aligned to the mm10 genome using *Bowtie2* (v2.5.4) with default parameters. Reads aligned to the genome were annotated for RNA types using *MGcount*⁴¹ (v1.1) with the mm10 annotation file.

Nascent tRNA analysis

The nascent RNA data comes from another manuscript prepared during the same period. Data were processed using *TrimGalore* (v0.6.10) to remove adapters and low-quality reads, followed by alignment to the mouse reference genome (mm39) using *HISAT-3N* (v2.2.1-3n-0.0.3) with parameters: --base-change A,G --unique-only --directional-mapping. Only properly paired reads that mapped in the correct orientation and within the expected distance of each other were retained for further analysis. To identify high-confidence 4sU-induced T-to-C mutations, the following filters were applied: (1) Sites with base quality <30 were discarded; (2) Sites within 5 bp of each read end were discarded; (3) SNPs identified using *VarScan*⁴² (v2.4.5) with the options --strand-filter 0 --min-avg-qual 30 --min-var-freq 0.2 --min-coverage 10 were discarded. For paired-end reads, mutations from both mates were counted together. Nascent reads were defined as those containing at least one T-to-C mutation, unless otherwise specified.

The tRNA annotation file for mm39 was obtained from *GtRNadb* (<https://gtRNadb.ucsc.edu>). *FeatureCounts* (Subread v2.0.8) was used with the options -p --countReadPairs -B -C -s 2 to quantify nascent tRNA reads within 100 bp upstream and downstream of tRNA genes, read counts were then normalized to RPKM. To avoid confounding signals, nascent tRNAs were further filtered by removing tRNA genes overlapping with exonic regions, merging read counts from three replicates, and excluding genes with stage 0. The filtered set was overlapped with expressed mature tRNAs. Heatmaps were generated using hierarchical clustering and visualized with the *ggplot2* (v3.5.1) package in R.

ChIP-seq, ATAC-seq data processing

Public ULI-NChIP-seq dataset²⁴ for H3K4me3 and H3K27me3 modifications on embryos from 2-cell stage to blastocyst stage (two or three replicates were performed for each stage) were obtained from GEO accession GSE73952. For ChIP-seq analysis, *Trim Galore* with default settings was used to preprocess ChIP-seq data. Reads were aligned to the mouse reference genome (mm39) using *Bowtie2* with parameters --no-mixed --no-discordant --no-unal, retaining only uniquely mapped reads. Reads with MAPQ > 10 were filtered using *samtools* (v1.6), duplicates were removed with *samtools* *markdup*, and replicates were merged. BAM files were normalized to RPKM using *bamCoverage* (-bs 10 --extendReads). Signal correction and conversion were performed using the *bdgcmp* module of *MACS3* (v3.0.2) with the -m subtract option to calculate the signal intensity difference between each ChIP sample and the corresponding Input sample, and bigWig files were generated (with a normalization bin size of 10 bp). Heatmaps were plotted using *deeptools*⁴³ (v3.5.5), with the tRNA gene start site as the reference point (--referencePoint TSS), using the nascent tRNA regions identified above (-R), and including 1 kb upstream and downstream flanking regions (-a 1000 -b 1000).

Public ATAC-seq dataset²³ were obtained from GEO accession GSE66581 on mouse preimplantation embryos from early 2-cell to blastocyst stage. For ATAC-seq analysis, *Trim Galore* with default settings was used to preprocess ATAC-seq data. Clean reads were aligned to the mouse reference genome (mm39) using *Bowtie2* with the

following parameters: up to one mismatch per read, the minimum seed alignment length to 25, specifying a maximum fragment length of 2000, retaining only alignments from the same fragment (-q -N1 -L 25 -X 2000 --no-mixed --no-discordant). PCR duplicates were removed using sambamba markdup⁴⁴ (v1.0.1) with default settings. Replicates were merged using samtools, and BAM files were normalized to RPKM using bamCoverage with a bin size of 10 bp. Heatmaps were generated using deeptools, with the tRNA gene start site as the reference point (-referencePoint TSS), using the nascent tRNA regions identified as described above (-R), and including 1 kb upstream and downstream flanking regions (-a 1000 -b 1000).

LC-MS/MS detection of modified nucleosides

Fifty oocytes or embryos were collected at each stage as one group, with two to three biological replicates, for total RNA extraction. Total RNA was digested in 20–40 μ L of solution containing 1 $\times$ Nuclease P1 Buffer, 0.2 U Nuclease P1 (New England Biolabs, M0660S), 0.2 U Quick CIP (New England Biolabs, M0525V), and 0.2 U Benzonase (Sigma-Aldrich, E8263) at 37 °C for 3 h. The mixture was passed through Nanosep with 3 K omega (PALL, FJ3290) and transferred to a 2 mL mass spectrometry vial (Agilent, 5182-0716) with a 200 μ L insert (Agilent, 5183-2089). Ten microliters of sample were separated using an Acquity UPLC I-class chromatography system (Waters, USA) with an Acquity UPLC HSS T3 1.8 μ m 2.1 $\times$ 150 mm column (Waters, USA) at 30 °C. Mobile phases were prepared and UPLC conditions were set as follows: mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. The gradient elution program was: 0–6 min, 0% B; 6–7.65 min, 0–1% B; 7.65–9.35 min, 1–6% B; 9.35–10 min, 6% B; 10–11 min, 6–10% B; 11–12 min, 10–20% B; 12–14 min, 20–50% B; 14–17 min, 50–75% B; 17–17.5 min, 75–0% B; 17.5–30 min, 0% B. The flow rate was set at 0.3 mL/min. A Sciex QTRAP 6500+ mass spectrometer (SCIEX, USA) was used in positive ion mode with multiple reaction monitoring (MRM) scanning. The parameters were set as follows: CUR: 30.0; CAD: 8.0; IS: 5500.0; TEM: 350.0; GSI: 30.0; GS2: 30.0. Each biological replicate was measured with one or three technical replicates. Mass spectrometry data processing, including spectra visualization, peak area calculation, and absolute quantification, was performed using SCIEX OS (SCIEX, USA). The ion pair parameters for the detection of 19 nucleosides are listed in Supplementary Table 1. Samples were absolutely quantified and calculated using gradient-diluted nucleotide standards, and the gradient dilution scheme for the 23 nucleoside standards is provided in Supplementary Table 2.

tRNA modifications analysis

For tRNA modification abundance analysis, the total abundance of a specific modification at a particular site was obtained by coverage weighting (misinc_proportion * cov). For tRNA modification heatmap analysis, only tRNA isodecoders with an average modification frequency of 1% or more across all stages were displayed, and different clusters were distinguished by k-means algorithm. The pipeline for crosstalks analysis of tRNA modifications has been integrated into the mim-tRNAseq pipeline, and the parameter "--crosstalks" was used in the mim-tRNAseq pipeline.

tRNA gene SNP analysis

To identify SNPs in all genomic copies of tRNA-Arg-TCG-3 and tRNA-Asp-GTC-1, primers were designed using Primer3Plus (<https://www.primer3plus.com>) within $\pm$ 400 bp of each gene (Supplementary Table 3). Genomic DNA was extracted using the EasyPure® Genomic DNA Kit (Transgene, EE101-11), followed by PCR amplification with GoTaq® Master Mix (Promega, M7122) under the following conditions: 98 °C for 2 min; 30 cycles of 98 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; followed by 72 °C for 5 min and holding at 4 °C. The purified PCR products were subjected to Sanger sequencing.

Codon usage analysis

The CDS lengths and codon usage frequencies for mouse mm39 genes were obtained from Ribotoolkit⁴⁵, and codon usage frequencies were calculated using all codons per mRNA. mRNA, RPF, and TE data for early embryonic development were obtained from Wei Xie et al.⁴. Anticodon proportions were converted from ORACLE-tRNAseq reads to the proportion of total tRNA reads. For wobble-paired codons, the anticodon proportion of the corresponding anticodon was duplicated and assigned to that codon, so that all 61 codons had corresponding anticodon proportion data. The weighted codon usage for a specified gene set was calculated using the following formula:

$$W_i = \frac{\sum_{j=1}^n (p_{ij} \cdot \exp_j \cdot \text{len}_j)}{\sum_{i=1}^m \sum_{j=1}^n (p_{ij} \cdot \exp_j \cdot \text{len}_j)} \quad (1)$$

Where m is the number of codons, n the number of genes, i the i -th codon, j the j -th gene, p codon frequency, $\exp$ mRNA expression or RPF, and len CDS length.

Codon occupancy analysis

Codon occupancy data were generated using the online tool RiboToolkit⁴⁵. Briefly, Ribo-seq raw data from Wei Xie et al.⁴ (GSE165782), were processed with cutadapt (v4.4) using the original parameters for adapter trimming, and converted into collapsed FASTA files, which were then uploaded to the RiboToolkit⁴⁵ server for analysis.

Gene set enrichment analysis

Gene Ontology (GO) enrichment analysis was performed for specific gene lists. First, we obtained GO annotation information for mouse genes from the Ensembl database using the biomaRt (v2.60.0) package in R. Then, we performed GO enrichment analysis on the gene lists using the clusterProfiler (v4.12.0) package in R. The enrichment score for each GO term was defined as the ratio of the proportion of genes annotated to that term in the target gene set relative to that in the background set, i.e., $(k/n)/(M/N)$, where k and n represent the number and total of genes in the target set, and M and N represent those in the background. GO terms with an adjusted P value (FDR) less than 0.05 were considered significantly enriched.

Statistics analysis

For two groups of samples, the Wilcoxon paired signed-rank test was used for paired samples, and the Mann-Whitney test was used for unpaired samples. For multiple groups of one-way paired samples, the Friedman test was used, and for multiple groups of one-way unpaired samples, the Kruskal-Wallis test was used. For biological duplicate samples, one-way ANOVA or two-way ANOVA was used. Multiple comparisons were adjusted using statistical hypothesis testing or false discovery rate (FDR) control. Correlation analysis was performed using the Spearman correlation coefficient. Linearity of the standard curve was determined by Pearson correlation coefficient. P value less than 0.05 was considered statistically significant.

Reporting summary

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

Data availability

The ORACLE-tRNAseq generated datasets and LC-MS/MS data are available in the National Genomics Data Center (<https://ngdc.cncb.ac.cn/>) under the BioProject number PRJCA038997. Other publicly available datasets used in this study have been obtained from NCBI's Gene Expression Omnibus with the GEO Series accession numbers GSE73952, GSE66581, and GSE165782. Source data are provided with this paper.

Code availability

Minor scripts for data processing and visualization are available at GitHub (<https://github.com/BIGRICEgo/ORACLE-tRNAseq>).

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Author contributions

Y.L. and Y.Z. conceived and designed the project. Y.L. developed the ORACLE-tRNAseq method and performed the ORACLE-tRNAseq experiment with the help of X.W., H.Y. and Z.C. X.W. collected oocytes and embryos with help from W.J. and Z.C. Y.L. performed the bioinformatic analyses of ORACLE-tRNAseq, ribo-seq, and proteomic data with help from Z.S., H.Y., Z.Y. and H.L. X.W. and W.J. performed the RNA modification detection experiments and analyses by LC-MS/MS with help from Y.L. R.H. performed the bioinformatic analyses of nascent RNA-seq, ATAC-Seq, and ChIP-Seq with help from X.-O.Z. and Y.G. Y.W. and R.Y. provided the embryonic nascent RNA-seq data. Y.L. and Y.Z. prepared most of the figures and wrote the manuscript with help from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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