Developmental regulation of Canonical and small ORF translation from mRNA

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Abstract

Ribosomal profiling has revealed the translation of thousands of sequences outside of annotated protein-coding genes, including small Open Reading Frames of less than 100 codons, and the translational regulation of many genes. Here we have improved Poly-Ribo-Seq and applied it to *Drosophila melanogaster* embryos in order to extend the catalogue of *in-vivo* translated small ORFs, and to reveal the translational regulation of both small and canonical ORFs from mRNA across embryogenesis. We obtain highly correlated samples across five embryonic stages, with some 500 million putative ribosomal footprints mapped to mRNAs, and compared them to existing Riboseq and proteomics data. Our analysis reveals, for the first time in Drosophila, footprints mapping to codons as the unequivocal hallmark of productive translation, and we propose a simple binomial probability metric to ascertain translation probability. However, our results also reveal reproducible ribosomal binding apparently not resulting in productive translation. This non-productive ribosomal binding seems to be especially prevalent amongst upstream short ORFs located in the 5' mRNA Leaders, and amongst canonical ORFs during the activation of the zygotic translatome at the maternal to zygotic transition. We suggest that this non-productive ribosomal binding might be due to cis-regulatory ribosomal binding, and to incomplete ribosomal scanning of ORFs outside periods of productive translation. Finally, we show that the main function of upstream short ORFs is to buffer the translation of canonical ORFs, and that in general small ORFs in mRNAs display Poly-Ribo-Seq and bioinformatics markers compatible with an evolutionary transitory state towards full coding function.

Introduction

Ribosomal profiling, the genome-wide sequencing of ribosome-protected RNA fragments (RiboSeq) has been increasing our understanding of a crucial event in all living genomes: protein translation[1, 2]. RiboSeq results show that there exists a noticeable level of non-canonical translation in eukaryotes. This can arise from non-AUG codons, from polycistronic transcripts, and from unannotated small Open Reading Frames of less than 100 codons (smORFs)[3-5].

At the same time, the comparison of RNA-Seq and RiboSeq for the same biological samples allows the transcriptome-wide study of an important but often forgotten

regulatory process in genome function: the regulation of translation. Often, expression of an mRNA transcript is equated with the automatic translation of any encoded canonical ORF (of more than 100 codons). However, translation is not automatic, but is regulated, often with great relevance for organismal function, both under normal and altered conditions [6-10]. Translation regulation is also a key aspect of development in all animals[11] and has been studied extensively in *Drosophila* [12-14]. *Drosophila* embryogenesis is a highly-coordinated and complex process that is completed in a time span of just 24 hours[15]. During the first two hours after egg laying (AEL) there is absence of transcription from the zygotic genome and the key developmental processes, such as establishment of the primary Antero-Posterior and Dorso-Ventral axes, are controlled purely through the translational regulation of maternal mRNA previously laid in the egg[16, 17]. After this initial period, the embryo undergoes a ´maternal to zygotic transition´, whereby the transcription and translation of the zygotic genome takes over the maternal products, a process also found in nematodes, echinoderms, and vertebrates[13, 18-20]. Nonetheless, the impact of translational regulation at the genome-wide scale on the whole of *Drosophila* embryogenesis has not been revealed.

RiboSeq results regarding non-canonical and regulated translation have been the subject of debate. While it has become accepted that both processes may occur more extensive than previously thought, there is no consensus on the actual fraction of smORFs and non-canonical ORFs whose translation is shown by RiboSeq[21-25]. The RiboSeq debate centres on the asymmetry between these numbers and other translational evidence, and on the interpretation of the RiboSeq results themselves. The most widely used counterpart of RiboSeq is proteomics, but the numbers of protein and peptides detected by proteomics consistently fall short of those detected by RiboSeq, especially regarding non-canonical translation. For example, the most thorough proteomics study to date covering the whole *Drosophila melanogaster* life-cycle has detectedless than 40% of all canonical proteins[26]. This number is further reduced to 30% of annotated smORF polypeptides, while we have previously reported that 80% of canonical and small ORFs show clear RiboSeq evidence of translation in a single embryonic cell line[23]. However, RiboSeq detects ribosomal binding, not actual peptide production. There is not a universally agreed RiboSeq metric unequivocally identifying productive, biologically-relevant translation, as opposed to other processes such as low-level background translation, ribosomal scanning and nonsense-mediated-decay surveillance, or stochastic ribosomal binding. Bioinformatically, it is accepted that

ribosomal binding above a certain level, and especially, binding showing tri-nucleotide periodicity in phase with codon triplets (phasing or framing), indicate translation of an ORF[1, 2].A biochemical approach is to introduce modifications to the ribosomal-RNA purification, to ensure that only ribosomes engaged in productive translation are selected. A tried approach is RiboSeq on polysomes (RNAs bound by several ribosomes), since the sequential translation of polyadenylated, capped and circularised mRNAs by several ribosomes is a feature of productive translation, and exclude single ribosomes (which could be involved in low-level translation but also in other activities) [23, 27].We have called this latter approach Poly-Ribo-Seq.

Here we present an *in vivo* Poly-Ribo-Seq study covering a time-course of *Drosophila melanogaster* embryogenesis. We have both improved our experimental Poly-Ribo-Seq and the subsequent data analysis pipeline, to obtain unprecedented levels of RiboSeq efficiency (reads mapped to ORFs) and quality, including codon framing as the hallmark of productive, biologically meaningful translation, which allows us to ascertain translation and its regulation *in vivo* and across development for both canonical and non-canonical ORFs. Thus, we detect the translation of thousands of non-annotated ORFs, and identify hundreds of mRNAs whose translation is highly regulated during embryogenesis. However, our results also reveal reproducible ribosomal binding not resulting in productive translation. This non-productive ribosomal binding seems to be especially prevalent amongst upstream short ORFs located in the 5’ mRNA Leaders, and amongst canonical ORFs during the activation of the zygotic translatome at the maternal to zygotic transition. We suggest that this type of ribosomal binding might be due to either cis-regulatory ribosomal activity, or to ribosomal scanning of ORFs outside periods of productive translation.

Results

1) The method and overall data

Since Poly-Ribo-Seq requires even larger amounts of starting material than Ribo-Seq due to polysome fractionation, the *Drosophila S2* cell line was an excellent tool for the development of the technique. However, *S2* cells do not provide a complete picture of *Drosophila* translation, as only 60% of both canonical genes and uORFs, and only about a third of annotated smORFs (hereafeter referred as short CDSs, or sCDSs) appear transcribed in this cell line[23]. The S2 cell line is derived from just one type of tissue (macrophage-like) from late stage *Drosophila melanogaster* embryos[28] and may not be the ideal system to obtain a comprehensive picture of *Drosophila* translation. Therefore, we applied Poly-Ribo-Seq to *D. melanogaster* embryos in order to extend the catalogue of *in-vivo* translated smORFs and study translation across three distinct developmental time windows of *Drosophila* embryogenesis. The original protocol from Aspden et al. 2014 was adapted and improved in terms of biochemical rRNA depletion and bioinformatics mapping of FPs to ORFs (see methods), and we obtained higher yield, purity and accuracy (Fig.1 and Sup. Table 1).This way we detected rare but reproducible translation events in non-annotated genes, and obtained a comprehensive picture of translation across development.

In our experimental design, we divided the *Drosophila* embryogenesis in three temporal 8 hour windows: Early (0-8 hours after egg laying, or 0-8h. AEL), Mid (8-16h. AEL) and Late embryogenesis (16-24h. AEL) (Fig. 1A). The key developmental processes occurring during these periods of embryogenesis are described elsewhere[15]. Briefly, early embryogenesis (0-8h. AEL) is characterised by the maternal to zygotic transition, followed by gastrulation, germ band formation and ectodermal and mesodermal determination and pattern formation, including the determination of the CNS. The 8-16h. AEL hours of development (mid-embryogenesis) covers the process of tissue formation and organogenesis (such as presumptive gonad and gut assembly) including multi-organ processes such as formation of segments and the feeding cavity. The differentiation of neurons and muscles also occur during this time. Late embryogenesis (16-24h. AEL) is characterised by final differentiation and the start of physiological processes such as epidermal and cuticle differentiation[29] (with opening of tracheae to respiration) and digestive system differentiation (with yolk digestion and uric acid) [30, 31], plus the

connection and fine-tuning of neural, sensory and muscle structures, eventually resulting in coordinated embryo movements[32, 33].

We collected two biological replicas (called T and B) from each embryonic stage, extracting simultaneously total RNA and Ribosomal Footprints. Sequencing and analysis of these samples was used to determine transcriptionand translation levels, which in turn were used to reveal the extent and quality of translational regulation during *Drosophila* embryogenesis at a genomic scale. We studied several classes of ORFs as defined in Couso and Patraquim 2017[34], altogether numbering40,852 ORFs: 21,118 canonical ORFs of more than 100 codons in polyadenylated mRNAs; 862 ORFs from annotated coding sequences of 100 codons or less in an otherwise canonical mRNA (short CDSs); and 18,872 upstream ORFs (uORFs) in the 5' leaders of canonical mRNAs. We studied only AUG-STARTORFs, and also discarded overlapping uORFs, in order to ensure the accuracy of our translation assessments. Similarly, the translation of ORFs in putative long-non-coding RNAs (lncORFs) will be described in a forthcoming study due to its special characteristics.

As the RiboSeq protocol typically results in a composite library with a majority of ribosomal rRNA reads, and a minority of ribosomal footprints (FPs), we sequenced an exhaustive amount, totalling1.2billion RiboSeq reads, yielding some 345million ribosomal footprints mapped to the genome (Sup. Table 1) with ~80% FP aligned to annotated CDS, ~ 16% to 5'Leaders and 6% to 3'UTRs (Figs.1B and 2A). The data shows high reproducibility, with high Spearman correlation(r=0.9) of RPKMs (number of sequenced reads per ORF length in kilobase,per million reads) for each ORF amongst the two replicas (Fig. 1C) for both RNA-seq($RPKM^{RNA}$)and RiboSeq ($RPKM^{FP}$) samples. Only reads of 26 to 36 nts were counted for $RPKM^{FP}$, as this length distribution corresponded to 99% of the reads obtained in all cases, and is within the size range previously described for FPs [1] (suppl.Fig 1A). At first glance, our data reveals translation of non-canonical genes, as for the polycistronic smORF gene *tarsal-less [35]*(Fig. 1D and Sup. Table 3A).

2) **Framing: an indicator of productive translation**

For our bioinformatic analysis (see Methods and Fig. 2A), we use $RPKM^{RNA}>1$ to indicate transcription of an ORF (in either of our two replicas).To determine translation, we use two combined filters. First, a quantitative filter: a minimal amount of ribosomal binding must be detected, set as a standard $RPKM^{FP}>1$ in both T and B replicas, to indicate signal above error and background. Second, a qualitative filter, the nature of that binding: the ribosomal movement across an ORF during translation generates tri-nucleotide phasing of ribosomal protected footprints, or framing (Fig.2B), in contrast with the reads derived from RNAseq, which accumulate evenly across all nucleotides in mRNA(Sup. Fig 1B Data).

Previous ribosomal profiling studies of *Drosophila* had not been able to observe strong framing on a genome-wide scale, and thus translation has been proposed on the basis of RPKM and other quantitative metrics, such as coverage[18, 23, 24, 36]. However, our improved Poly-Ribo-Seq method, coupled with an improved bioinformatics analysis of the data (see methods), revealed framing for the first time in the *Drosophila* (Figure 2B-C). Likeall RiboSeq experiments, our data revealed a distribution of Ribosomal footprint lengths (Sup. Fig 1A). We focused on the most represented read length (32 nucleotides), which also shows the clearest framing in all samples (55-60% reads in-frame; Figure 2C). Different formulae and metrics have been previously used to quantify framing per ORF and provide a cut-off[1, 21], reviewed in [5, 37], which was usually obtained by sampling a small number of bona-fide translated ORFs and extrapolating the results. While these methods can identify translation, they can be complex and difficult to apply universally to all RiboSeq datasets, due to differences in sampling and protocols utilised.

Here, we have opted for a simpler approach that is easy to extend to past and future datasets. We treat framing as a simple coin-toss problem, which offers clear statistical probabilities, even with a low number of reads. As each footprint is the result of an independent RNA-ribosome molecular interaction, each resulting sequencing read has in principle a 1/3 probability of aligning to the right frame in an ORF, and 2/3 of aligning to the wrong frame (Fig.2D). The probability of having x successful outcomes (reads aligned to the ORF frame) following n independent trials (number of reads mapping to the ORF) follows a classical binomial distribution, which can be consulted using widely available tables [38] and computer programs. We take the observed binomial probability as the probability of being translated for a given ORF and we consider it as indicative of translation when $p<0.01$ (i.e. the probability of observing such framing of reads in the

ORF by chance is less than one in a hundred; see Methods for details and implementation).Here, because of our $RPKM^{FP}$>1 filter, and because only 32ntFP reads were used to calculate framing (see methods and Sup.Fig.1) we noted that no ORF appeared translated (`framed´)having less than 30 ribosomal footprints (of 26 to 36nts) mapping to it. As example we show the non-canonical translated smORF in the *sarcolamban* (*scl*) gene[39],where most of its FP reads mapped to the 2nd frame and displayed a binomial *p*-value of 7.1995E-52,corroborating the translation of the *scl* ORF A (Fig1E).Note that framing *p*-values do not indicate the amount of translation (which would be indicated by the $RPKM^{FP}$ and its associated metric translational efficiency, TE, see below) but only the likelihood that the observed ribosomal binding is due to ORF translation. This framing metric also ensures that the translation detected belongs to a given ORF, not to another putative ORF overlapping it.

Using these criteria we observed that 98% of transcribed canonical ORFs, 90% of short CDSs and 72% of uORFs were reproducibly bound by ribosomes at any point during development (i.e. showed RPKM>1 in both replicates), whereas the percentage of actually translated ORFs (framing p-value<0.01) was 92% of Canonical, 73% of short CDSs, and 13% of transcribed uORFs (Fig. 2F).

3) Consistency of Transcription and Translation measurements across different types of genomic data

We further verified the robustness and reproducibility of our data and analysis by cross-comparisons across related gene-expression genomic data: RNAseq, Ribosomal profiling, and quantitative proteomics. For RNA expression, we consolidated the modENCODE embryo RNA-Seq data [40], into our three 8-hour long developmental time windows. For all three developmental windows, the RPKM of RNA-Seq samples was more highly correlated (in the region of *r*=0.9) with a replica of their stage (either ours or modENCODE), than with samples of other stages (Fig. 1C, Fig. 3A, Sup Table 2).

For Ribosomal profiling, our $RPKM^{FP}$ data showed an even more marked trend to correlate preferentially first with their own stage replica (r=0.98-0.99), and second with samples from adjacent time periods (Fig. 1C, 3A, Sup Table 2). Our FPs and RNA-Seq from the same stage showed a high correlation of r=0.8-0.9 (Fig. 1C, Sup Table 2). We

also compared our FP data with the Kronja et al. 2014 RiboSeq data from unfertilized *Drosophila* eggs,that was obtained after RNaseI digestion akin to our own protocol. As expected, the $RPKM^{FP}$ from unfertilized eggs (FP Maternal) showed the highest correlation with our $RPKM^{FP}$ samples from early embryogenesis (0-8h AEL) (Spearman´s $r \approx 0.8$) (Fig. 3A and Sup. Table 2).Our bioinformatic pipeline detected framing in Kronja´s ORF-mapping reads of 29nt (see methods). 10,314 canonical ORFs appeared translated both in unfertilized eggs and early embryos, albeit showing increased translation efficiency in the embryos (Fig. 3B).However, differences between the maternal and zygotic translatomes were also identified, since Kronja only detects maternal RNA translation while our 0-8h AEL sample detects both maternal and early zygotic translation (see “Regulation of translation” below).

We also compared our embryo FP data with *Drosophila* embryo-derived S2 cell cultures[28], which in principle should provide synchronic data less subjected to experimental and developmental noise. We have re-analysed the data from Aspden et al. 2014, adding a new Poly-Ribo-Seq sample obtained here. S2 translation in general is highly correlated with embryonic one (Fig. 3A), and the proportions of canonical ORFs ribosome-bound are 98%and translated 63%. Despite this overall similarity, differences were also noted (see “Regulation of translation” below).

For proteomics, we consolidated the results of extensive quantitative proteomics across embryogenesis[26] in our three 8h developmental time windows (methods). This quantitative proteomics data showed an overall 0.45correlation with our $RPKM^{FP}$ values (Fig. 3C). This correlation appears modest, but it is highly significant (at $p<0.0001$) indicating that $RPKM^{FP}$ can be a good proxy of protein-producing, productive canonical translation. However, 40% canonical ORFs detected as transcribed by RNA-Seq by us and modENCODE during embryogenesis are not detected by proteomics (Fig. 3D). The median $RPKM^{FP}$ value of the proteomics-detected proteins (32.11) is much higher than for those not detected (13.41), indicating that proteomics detection needs high levels of protein translation, as noticed previously [21, 23]. Yet, many ORFs with experimentally-verified translation, and with high Poly-Ribo-Seq metrics, are not detected by proteomics, including all *Hox* genes except the more widely-expressed *Ubx* and *abd-A*. This `proteomics detectability´ issue is not attributable to trypsination treatments, since we observed that 99% of canonical and 92% of non-canonical ORF could produce K- and R-cleaved peptides of 7 to 24aa-long suitable for mass-spec (MS) detection. Hence,

other yet unknown factors must influence the proteomics detection of proteins. In addition, small peptides of less than 50 amino acids suffer from enhanced degradation[41, 42], further hindering their detection by proteomics. Indeed, we observed that sCDSs are especially handicapped for MS detection, since the median $RPKM^{FP}$ of those detected is 172.1(Fig. 3E). Finally, when comparing MS data with RNA-Seq and Poly-Ribo-Seq, and contrary to that observed between RNA and FP data (Fig. 3A) the highest correlation observed is amongst MS data from different embryonic stages. Altogether our analysis suggests that proteomics is good at detecting a set of constitutively and highly translated proteins, but not so good at detecting ORFs translated either moderately, or in a developmentally-regulated manner.

smORFs also appeared harder to detect by Poly-Ribo-Seq than canonical ORFs, requiring a median $RPKM^{FP}$ of 40.5 (Fig. 2F, 3E). As an independent indicator of translation, we have tagged a sample of smORFs from sCDSs and added to the results from Aspden et al. 2014, for a total of 45 sCDSs. We compared the results of tagging with our pipeline and with S2 cell proteomics data [43]. Out of the 45 tagged sCDSs, we observed that 32 were not detected by S2 cells proteomics, 19 by our Poly-Ribo-Seq, and 5 by tagging. Four out of these five peptides not reliably detected by tagging were also not detected by Poly-Ribo-Seq, and 17 of the peptides not detected by Poly-Ribo-Seq were also not detected by proteomics. Thus the three techniques, based on very different technologies (Mass-spec vs. NextGenSeq vs. Confocal microscopy, respectively), show increasing sensitivity thresholds: Proteomics <Poly-Ribo-Seq< Tagging. However, our expression of tagged peptides in S2 cells is based on non-endogenous high transcription and this might have pushed the peptide production of lowly expressed ORFs over the edge of detection (see methods and Aspden et al. 2014). Thus, our tagging results may indicate what “can be translated” rather than what actually “is”. The most abundant tagged peptide expression was reticular-punctated in the cytoplasm (Fig.3F), which may correspond to mitochondria[23], or else to mitochondria-associated ER producing the peptide. An organelle localisation could fit with the postulated tendency of the sCDSs class as a whole for membrane-associated localisations [23, 34].

4) Regulation of canonical and sCDS translation during embryogenesis

According to our data and modENCODE, 82% of canonical ORFs and 57% of short CDSs were transcribed at any point during development (Fig. 2F). Of these, 8% and 27% respectively were never translated during embryogenesis, giving a first indication of the extent of translational regulation in *Drosophila melanogaster*.

Our data also showed that translation of some 80% of canonical ORFs was detected constitutively (at all embryonic stages), whereas 20% was detected only at specific stages of development (Fig. 4A). Stage-specificity seemed to be higher for smORFs, since only57% of short CDSs and 19% of uORFs were detected across embryogenesis, whereas 43% and 81% respectively were translated only during some stages of embryogenesis. However, this developmental analysis reflects both transcriptional and translational regulation. To further quantify the extent and dynamics of purely translational regulation, we analysed the changes in their translational efficiency (TE) across stages (TE indicates the ratio between Ribo-Seq and RNA-Seq signal, calculated as $RPKM^{FP}$ / $RPKM^{RNA}$). This TE analysis revealed both on-off translation changes, and modulations of sustained translation, from one embryonic stage to the next. The average TE varied somehow across development (Fig. 4B), but in general we observed fairly stable and correlated TE values across development (but see also “uORFs as translational regulators”). To identify which ORFs undergo statistically significant translational changes we used the Z-ratio method (which basically indicates whether the variation of a given ORF TE is significantly outside the standard deviation of the total sample;see methods) [44-46]. We also included in the analysis the Unfertilized Egg and S2 cells TE data (comparing them to Early and Late embryogenesis, respectively). We observed significant translational changes in all ORF classes, with the percentage of changed ORFs depending on the ORF class.About12% of canonical proteins (1,904of 16,017 analysed), 16% of short CDSs (57 of 358) and 18% of translated uORFs had significantly changed TE from any given stage to the next, indicating significant translational regulation (Fig. 4C). One of the most abundant changes was the translational up-regulation of539canonical ORFs from early to mid-embryogenesis (Fig. 4D). These ORFs were enriched for GO expression terms indicating a role in organogenesis i.e. CNS, epidermis and digestive system development (Fig.4E), as befits the developmental processes underway (Fig. 1A).

The comparison of unfertilized eggs with 0-8h AEL embryo data revealed the maternal to zygotic transition in the translatome. The degradation of maternal mRNAs after the

zygotic to maternal transition from 2h AEL, and their substitution by zygotically-transcribed mRNAs has been reported, but a number of mRNAs do persist during early embryogenesis (Stephan and Claudio). Our results suggest that some of these maternal mRNAs that persist are subjected to translational repression as an added layer of gene regulation, whereas others go on to increase their translation during embryogenesis. Applying Z-ratio analysis, we observed in total 374 canonical ORFs with significantly changed translational efficiency between the maternal and the early zygotic translatomes (Fig. 4C), of which 237 where down-regulated (including 58*Dscam* ORFs) and 137 up-regulated, showing RNA expression patterns preferentially registered as ´maternal´ or 'ubiquituous´ at this stage (Fig. 4D,F).Further, the maternal to zygotic transition in translation was also highlighted by the correlation between qualitative and quantitative changes in ribosomal binding. 259 maternal-only canonical ORFs appeared translated (framed) in eggs but not in 0-8h AEL embryos (Fig. 3B), undergoing a significant reduction in ribosomal binding efficiency during this period (as shown by TE, Fig. 3B insert). Reciprocally, 232 ORFs were just ribosome-bound in oocytes, but fully translated in early embryos, in correlation with an increase in their TE (Fig. 3B, insert).Finally, 3,170 ORFs were translated in our 0-8h AEL samples but did not yield ribosomal FPs in eggs, presumably corresponding to newly-expressed, zygotic-only ORFs (Fig. 3B).

We hypothesized that a detailed comparison of late embryogenesis (from where the S2 macrophage-like line was isolated, (I. Schneider) translatome with that of S2 cells might reveal the S2 cell-specific translation profile. We observed 247 ORFs translated in S2 cells but not in late embryos, whereas 7,628 ORFs appeared translated in late embryos but not in S2 cells (Sup. Fig.2). In addition, 541ORFs showed significantly lowered TE in S2 cells and 350significantly raised TE (Fig. 4D). In principle, these differences might reveal a macrophage-like regulatory state, an adaptation to culture, or just a sampling issue (see discussion). Interestingly, we note that the S2 FPs correlate more closely with Early than with Late embryogenesis FPs (Fig. 3A), perhaps reflecting a 'de-differentiation' of the S2 macrophage precursors in cell culture.

5) uORFs as translational regulators (Figure 5)

We obtain highly reproducible Ribo-Seq signal average for uORFs across biological replicates in the Embryo (r=0.95, Sup. Fig. 3A), indicating that most uORFs are bound by ribosomes at specific levels across embryogenesis. Indeed, a majority (72%) of

embryo-transcribed uORFs show reproducible ribosome binding during embryogenesis ($RPKM^{FP}$>1 in two replicates, Fig. 2F). However, only 21% of these ribosome-binding events shows reliable translation signal, as measured by the binomial framing statistic (Figure 2F), suggesting that a majority of uORFs in *Drosophila melanogaster* might not have a peptide-productive role.

Eukaryotic uORFs can act as cis-translational repressors of a canonical main ORF (mORF) located downstream in their transcripts (Kozak). This regulatory role has been extrapolated to the transcriptome-wide level as the main function of uORFs [47-50]. We have studied the possible cis-regulatory effect of uORFs. Among canonical protein-coding genes, we see significant shifts in translational regulation across embryogenesis in hundreds of ORFs (Z-ratio ≥ |1.5|) in both Early-to-Mid (705 ORFs) and Mid-to-Late Embryogenesis (722 ORFs; Figure 4B). However a much smaller number of uORFs exhibited variation in Z-Ratios of translation (147 and 130 uORFs, respectively; Figure 4C), therefore it seemed unlikely that translated uORFs underpinned the translational changes in canonical ORFs. However, the cis-regulatory role of uORFs is in principle based on ribosome binding and it is mostly independently of the peptide being produced[51-53], so it could conceivably be carried out by non-productive ribosomal binding too. Therefore, we extended our analysis to include non-framed, ribosome-bound-only uORFs as well (i.e. $RPKM^{FP}$>1 but framing p>0.01; Fig. 2A), since this provided a combined pool of 8,504 ribosome-associated uORFs (Fig. 4C).

Previous genome-wide studies in Zebrafish[48] and across vertebrates [49] have found a negative correlation between the number of uORFs within a 5'-leaderand the TE of the main ORF. We wondered if this was extensible to our 8,504 ribosome-associated uORFs. This would indicate that this effect is indeed mediated by uORF ribosomal activity and not a function of other 5´leader features [49]. However, we do not see an effect of ribosome-associated uORF number on mORF TE (Figure 5A). Instead, we observe a stable TE across mORFs carrying different numbers of uORFs in their 5´Leaders.

Still, it would be possible that a minority of uORFs might have important cis-effects on their mORFs. We looked for this 'uORF onto mORF' regulation by asking if the 1,037 uORFs with significantly changed TE across stages (270 framed and 767 ribosome-bound-only, Fig. 4C) also show simultaneous significant TE changes in their cis-

associated mORF. We found that not to be the case: 79% (817 of 1,037) of uORFs significantly regulated do not have mORFs with significantly changed TE Z-ratios (≥ |1.5|), and vice-versa (Figure 5B). Yet, 12uORFs correlated negatively with their canonical ORF (i.e. as the uORF TE went up the mORF TE went down, Fig. 5B), suggesting a negative cis-translational regulatory role for these uORFs. Another 25 uORFs displayed reduced TE while their respective mORF TEs were upregulated, which is compatible with such a negative regulatory role. However, 183 uORFs showed coordinated changes with their mORF (i.e. uORF and mORF went both up (124) or both down (59) significantly, Fig. 5B), and this positive correlation extended to the TEs of all 8,504 ribosome-associated uORFs and their mORFs (r=0.47 Supp. Fig. 3B). This positive correlation could indicate that uORFs act as either: a) positive translational regulators; b) constant 'brakes' or negative regulators that reduce but does not overcome mORF translation; or finally, c) passive `bystanders´ that are ribosomal-bound by virtue of being present in the 5´Leader of a transcript containing a highly translated mORF. In addition, a combination of these roles is also possible. Interestingly, the total range of TE variation diminishes as the number of ribosome-bound uORFs increases within a given mORF 5' Leader (Fig. 5B), with canonical ORFs which contain no uORFs in their 5´-leaders displaying twice the coefficient of variation as those with one or more uORFs (Fig. 5B, inset). This suggests accumulative role for uORFs in the maintenance of translational efficiencies, possibly acting as buffers of variation in mORF translation. A buffering effect could also explain the higher TE averages amongst uORFs than amongst canonical ORFs (Fig. 4B), even though the proportion of ORFs with significantly changed TE was identical (1,037 of 8,504 uORFs: 12% vs. 12% canonicals, Fig. 4C - but see also below).

We observed that 45% percent of canonical ORF 5'-Leaders contain uORFs, a number in line with observations across metazoans[25, 34, 54]. Interestingly, this number is significantly lower in shortCDSs (25%). This could be explained by the difference in 5´ Leader lengths between these two classes (306nt versus 194nt). Indeed, the number of uORFsis positively correlated with the length of the 5´ Leaders across all annotated mRNAs (r=0.668****,Fig. 5C), indicating that uORFs tend to accumulate more often in longer 5´-Leaders. The positive correlation between uORF number and 5´Leader length supports the idea that uORFs appear randomly in 5' Leaders [34].

Whether uORFs act as cis-translational regulators, an important number (1,489, Fig. 2E) show framing, i.e. appear to be translated into peptides. On average, RPKM (Sup. Fig. 3C) and TE (Fig. 4B) was higher in these framed uORFs, and changed more dramatically across stages than on ribosome-bound-only uORFs (note steeper slope in Fig. 4B and higher total percentage of significant translational regulation inFig. 4C). The regulation of translated uORF expression was not only quantitative, but included qualitative changes as well (i.e. from ribo-bound-only to framed, correlating with an increase in 32nt FPs mapping to the uORF), and transcriptional regulation, altogether producing that 81% of uORFs were translated in a stage-specific manner (Fig. 4A). Thus, regulated expression seemed a feature of uORF translation.

In the absence of MS data to corroborate whether framed uORFs do indeed produce peptides, we checked other bioinformatics markers of coding potential. We observed that translated uORF start codon contexts show significantly better Kozak scores [52] than ribosome-bound-only uORFs (Fig. 5D), further indicating these are decoded by elongating ribosomes rather than just scanned through by small ribosomal subunits. Further, the sequence conservation of translated uORFsis intermediate between ribosome-bound-only uORFs and canonical ORFs (Figure 5E). The amino acid usage produced a similar picture (Figure 5F). These observations might suggest that translated uORFs either produce a specific type of peptide, or that translated uORFs are in an evolutionary transition into coding-ness [34]. One end result of such a transition would be to produce a di-cistronic coding gene (uORF plus mORF), and it is interesting that genes annotated as such by FlyBase display similar conservation to canonical ORFs (Fig. 5E), but aminoacid usage intermediate between these and translated uORFs (Fig. 5F). Tagging of selected translated uORFs and dicistronic peptides corroborated their translation and displayed similar subcellular localisations as sCDS peptides (Figs. 5G,H and 3F).

DISCUSSION

Translation vs. peptide function: Protein translation is the most expensive gene activity undertaken by a cell, one or order of magnitude more costly than either replication

or transcription, and costly enough to have a selective impact [55]. However, it is important to disentangle the act of productive translation from its biological purpose. It is usually expected that translated ORFs must produce a peptide or a protein with canonical function. However, a translated peptide could have other biological activities, [56] or no peptide function at all, such as the act of translation could be the biologically relevant function of the encoding ORF (Ribosome fidelity or process ability /Surveillance of transcripts mutations NMD triggering)[51, 57] . RiboSeq, and other techniques such as proteomics, do not reveal protein and peptide function, only its expression. Protein function is suggested by other studies (such as sequence conservation) and ultimately tested experimentally.

smORF translation is difficult to ascertain, but has been repeatedly detected to occur at a large scale by Ribo-Seq, including in this study. Different types of studies and analyses yield numbers always in the thousands per genome [58]. These numbers are a small fraction of the smORFs in the genome (about 5%), yet it is large enough (about a 10% of extra coding genes to add to canonical genes) to present a challenge to our understanding of genomes and their function. What can be the biological purpose of this 10% ´crypto-translatome´? Due to their shortness, it is challenging to determine smORF function from their sequence. GO analysis of the longer, and annotated, sCDS smORF class, and analyses of their aminoacid composition[23, 34], suggested roles related to cell membranes and organelles. However, shorter smORFs such as uORFs and those found in lncRNAs (called lncORFs) are not annotated and do not display known protein domains, nor homologies to characterised proteins, that could suggest a peptide function. Their AA composition is intermediate, yet distinct from canonical proteins and random sequences[34]. Nonetheless, important functions for lncORF and uORF peptides have been proven experimentally (OUR REFS) in a few cases, as well as a cis-regulatory, ‘non-coding’ function for uORFs [51, 53].

Ribosomal binding versus productive translation: Our results suggest a distinction between ribosomal-binding-only and productive translation. We observed that about 82% of uORFs show abundant, yet not framed, ribosomal binding. This is unlike canonical ORFs and short CDSs, where 95% and 81% respectively of the detected ribosomal association corresponds to productive translation. This raises the possibility that either much of ribosomal binding in uORFs serve purposes other than peptide production, or that it reflects ‘translational noise’. In view of the energetic and selective

costs involved, it is unlikely for the cells to allow for their resources to just be ´lost in translation´. An explanation might be suggested by the maternal translatome.

Maternal Translation

The maternal to zygotic transition corroborates that ´ribosomal-binding-only´ without framing is a different state than fully-fledged translation. Our data revealed ORFs ´poised´ for translation between unfertilized eggs, and Early embryos. 232 canonical ORFs switch from unproductive ribosome-bound-only to productive translation, while increasing their translational efficiency (Fig. 3B). In other words, the quality of their ribosomal association changes from low affinity (low TE) and not following a particular frame, to a higher-affinity, frame-linked one. To produce non-framing, ribosomal binding must happen in overlapping frames, or ´shift´ between frames, or include ´scanning´ 40S subunits, or proofreading ribosomal units involved in NMD and not productively reading codons. Interestingly, we observe one-frame shift of ribosomes at STOP codons (from blue to red, Fig. 2B,E), also noticeable in other Ribo-Seq studies [21], which probably reflects a conformational change or the ribosome pausing for a time at its A site while disengaging from the mRNA. Repeated cycles of ribosome assembly at START codons and disassembly at the ORF, before the STOP codon, as observed in uORFs when elongation stalls [59] (perhaps due to a failure to acquire AA-loaded tRNAs or elongation factors) in the absence of strong translation could distort the main framing signal. In other words, ribosome-bound-only ORFs could be failing to produce enough start to stop translation to overcome a low level and noisy ribosomal binding. This effect could be temporary (as in the maternal to zygotic transition) or a constitutive feature of the ORF (as in most uORFs, see below). Interestingly, we observe that STOP-like “red” framing in ribosome-bound-only uORFs is as prevalent as “blue” (proper) framing.

Function of uORFs

In our previous study [23] we estimated that 34% of Drosophila uORFs were translated in S2 cells, on the basis of a high $RPKM^{FP}$. Interestingly, our improved methods reveal two uORF pools, with highly overlapping $RPKM^{FP}$, yet different averages, and differences in framing (Sup. Fig. 3C). The percentage of ribosome-bound uORFsin *Drosophila* embryos(72%), is much higher than previously estimated, but the percentage of actually translated uORFs in our samples is 13%. The significance of this non-productive ribosomal-binding-only is intriguing. The positive correlation between uORF and mORF

with significantly changed TEs, the genome-wide lack of effect of ribosome-bound uORFs on mORF TE, and the correlation between 5´Leader length and number of uORFs all seem to suggest that most uORF are a random and function-less by-product of 5´Leader sequence variation, also suggested by the random-like size distribution of uORFs [34]. Yet, this does not exclude that some uORFs could have a cis-regulatory, or a peptide-producing role, as the examples shown. Different mechanisms have been proposed to mediate a cis-regulatory function, including ribosome stalling and disengagement at uORFs, a reduction in translational re-initiation on the downstream mORF AUG codons, of the triggering of mRNA decay [52, 53, 60, 61], all leading to a decrease in mORF translational efficiency. A positive regulatory role is also possible, with uORFs acting to recruit ribosomes and thus increase the likelihood that they reach mORFs via reinitiation. We propose a combined role, with most uORFs acting as ´translation buffers´ that would recruit ribosomes (a relatively scarce yet valuable cellular resource, Warner, J.R. 1999), and pass them on to their mORF at a constant rate, while discarding any excess. A passive, low-level uORF buffer effect at the genome-wide level is consistent with studies in yeasts [62, 63] and vertebrates [25] where a genome-wide positive correlation between the TE of individual uORFs and their cognate mORFs is also observed.

A mildly beneficial buffering role could explain the ubiquity of uORFs in otherwise canonical mRNAs. The minority of uORFs showing peptide-productive translation could be a tolerable by-product of this buffering role, which would otherwise be mostly mediated by the much more prevalent uORFs with non-productive ribosomal-binding-only. Interestingly, we observe subtly different Kozak scores, conservation levels and aminoacid usage for translated uORFs, which seem to fit into a stepped continuum ranging from transcribed-only uORFs to canonical ORFs. It is tempting to speculate that, just as ribosomal-bound-only in canonical ORFs may be a developmental transitory state during the maternal to zygotic translation, ribosome-bound-only uORFs may be an evolutionary transitory state poised for a transition to full coding function. In this way, the 5´Leaders of canonical genes would act as ´small ORF nurseries´ offering a tolerant environment for random uORF creation, while providing coding mRNA features such as poly-A tails, splicing-related and translationally-related RNA processing and stability, allocation to polysomes, and a specific transcriptional pattern. Dicistronic genes could be another step in this transition from inert or regulatory uORF to coding ORF (although dicistronics can also emerge from the fusion of two pre-existing canonical ORFs).

Interestingly, short CDSs, which in most aspects studied here (RPKM reproducibility; translation percentage; MassSpec detection; embryonic stage specificity; prevalence of translation regulation; amino-acid usage) are intermediate between translated uORFs and canonical ORFs, do not show intermediate conservation (Fig. 5E). The lower sCDSs conservation may suggest that sCDSs are also involved in another process, perhaps another and more dynamic stepped continuum leading to coding-ness via lncRNAs [34].

Further developments- single cell translatomics

Despite the improvements to our Poly-Ribo-Seq protocol, and the large number of reads that we have collected, it is possible that not all embryonic translation has been detected in our samples. Lowly abundant or transient mRNAs may not be adequately detected in our 8-hour developmental windows, or may be drowned out by noise and not be able to achieve RPKM >1. This could be especially true of mRNAs expressed in a few cells of the embryo, a situation that must arise often towards the end of embryogenesis, and could be prevalent in differentiated tissues. Our macrophage-like S2 cell line data allows for an approximation to this issue, and the problem of integrating and comparing Ribo-Seq and genomic data from whole embryos and cell lines. Direct comparison between late embryos and S2 cells creates a sampling issue. The embryonic counterparts of S2 cells could be only a few cells amongst many embryonic cell types and some 30,000 cells overall [64, 65]. Thus, the FPs from S2-related ORFs might not be adequately detected in whole embryo extractions (thus yielding a low or no RPKM), whereas the same FPs will be well-detected in the monotypic S2 cell culture. For example, we showed the specific expression (by *in situ* hybridisation and RT-PCR) and function (by observation of mutant phenotypes in embryonic macrophages) of the *hemotin* gene in the *Drosophila* embryo [66], yet our RNA-Seq and Ribo-Seq data, and modENCODE RNA-Seq would indicate that *hemotin* is neither transcribed nor translated during embryogenesis, while the same data show vigorous expression in S2 cells ($RPKM^{FP}$= 58.6). Altogether, this sampling issue can contrive an artefactual increase of expression and translation (RPKM and TE) of *hemotin* and similar cell-specific transcripts in cell lines [67]. However, a lowering of RPKM or TE in S2 cell cultures in 541 ORFs (Fig. 4D) can´t be due to this sampling issue, and must reflect specific translational repression in a S2 ´cell fate´, following either a macrophage-like fate, or a cell-in-culture fate. Currently, the demand for high levels of starting material (in the region of µg) is the largest barrier to single-cell RiboSeq, but it could be overcome by the combination of Ribosomal

Immunoprecipitation (TRAP), (which requires even more starting material (in the region of mg, [68, 69], with further improvements to the core RiboSeq protocol [70].

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MATERIALS AND METHODS

Ribo-Seq and RNA-seq procedures.

The RNA-seq and Poly-Ribo-Seq experiments were conducted as previously described in Aspden et al, 2014 with the following modifications. Embryos were flash frozen, turned into powder using a pre-chilled pestle and mortar and homogenized in Lysis Buffer at 4C for 20 min. Pre-clarified lysates were processed for mRNA isolation and fragmentation and Polysome separation and digestion as previously described. Digested polysome samples were concentrated by centrifugation and subsequently loaded into a 1M Sucrose Cushion and centrifuged at 70,000g to pellet the monosomes. The 50nt fragmented mRNAs and 28-34 nt footprints were isolated from a10% denaturing acrylamide gels and subsequently T4 PNK treated for library preparation.

The NEBNext Small RNA Library Prep Set for Illumina (NEB, E7300) was used following manufacturers' instructions with small modifications as follows. After the 3'Adapters ligation step, ribosome footprints were rRNA depleted using biotinylated DNA fragments and oligos from rRNAs and going through two consecutive rounds of subtractive hybridisation (except for the T 0-8h sample having only one round) using MyOne Streptavidin C1 Dynabeads (Invitrogen). After Ethanol precipitation, samples were

processed as NEB guidelines. Libraries were isolated from 10% non-denaturing gels by size selection and their quality and quantity measure by DNA high sensitivity chips (Agilent) with Bioanalyzer and Qubit.

Footprint sequence alignment

Ribosomal Footprints were filtered (PHRED quality≥33) and clipped for adapters using the FASTX-Toolkit. The resulting reads wer ealigned to a FASTA file containing rRNA, tRNA, snoRNA and snRNA r6.13 annotations using Bowtie, discarding the successful alignments and collecting all unaligned reads. Unaligned reads were then mapped to the FlyBase *D.melanogaster* reference genome (Release 6.13) using HISAT2 with default options. Due to the very low frequency of multimapping reads, we retained all genome-aligned reads for further analyses.

ORF selection and identification

All annotated coding sequences (CDSs) in FlyBase, r613 were retrieved and divided into groups of longer than or shorter than 303 nucleotides (100 AA). CDSs longer than the cut off were assigned to the canonical ORF category. CDSs shorter or equal to 303nt. in length, but arising from the same locus as canonicals were discarded from this analysis (short-isoforms). The remaining short CDSs, which arise from independent genomic loci, were assigned to the shortCDS category. We identified uORFs longer than 10 aa with an AUG start codon followed by an in-frame stop codon within the annotated 5′-Leaders of all annotated transcripts in FlyBase r6.13, using the emboss getorf program. All ORFs with fully-redundant CDS coordinates were identified, and duplicates were discarded within each class (canonicals, shortCDSs and uORFs), Additionally, we only kept uORFs with no overlap with any annotated CDSs. uORFs in dicistronic transcripts were then added to the uORF set, based on a full overlap with annotated ORFs contained within the 5’ Leader of an immediately downstream annotated CDS.

Ribosomal Footprint analysis

Relative Ribosome density ($RPKM^{FP}$) was measured by counting Ribo-Seq reads overlapping each feature using the R package Rsamtols (absolute ribosome density), and scaling this number by feature length and the total number of genome-aligned reads

[2]. Total mRNA expression ($RPKM^{RNA}$) was ascertained using the same features and applying the same method on RNA-Seq measurements. Correlations analyses across replicates were measured by Spearman´s rho on ORF RPKM values.

Detection of framing and individual translation events

To analyse framing, ribosome footprints (FPs) were first aligned to an artificially constructed transcriptome set, consisting of all ORFs analysed in this study as well as their surrounding regions -18 nucleotides upstream and +15 nucleotides downstream, thus allowing for the alignment of full ribosomal reads spanning the START and STOP codons. The resulting alignments were analysed using the R package RiboSeqR to extract the global framing patterns across ribosome footprint lengths for three pools of RPFs: all pooled embryonic RiboSeq samples, S2 cell samples and pool of mature oocyte and activated eggs RPFs (Kronja 2014, cicloheximide^{+}). The main read length and its most overrepresented frame in each sample was then used to evaluate the framing of each ORF in each Ribo-Seq sample. For each ORF, we then compared successful framing events (those matching global framing patterns) with unsuccessful framing events using the binomial test implemented *pbinom()* function in R to measure the probability of obtaining the observed framing (or better), when compared to the expectation by random (1 in 3 probability). For binomial tests of ORF translation, different biological replicates (if available) were merged into a single one. Although the precision of the binomial probability method improves with on the amount of evidence (i.e. higher number of reads can achieve very low p-values, or very high probabilities of being translated, Fig. 2C), it can still achieve results with minimal information, which is especially relevant for smORFs as their short size accrues less reads in sequencing experiments when compared to canonical ORFs with the same RPKM range (Fig. 1, 2E). Thus, a minimum of 5 reads is required to obtain $p<0.01$ if all reads are in frame, or more than 7 reads if any is out of frame [38].

Comparison with other RiboSeq and RNA-seq datasets

We obtained previously-published [18] fastq files containing Cycloheximide+ RiboSeq reads from mature oocytes and activated eggs from the NCBI Sequence Read Archive (accessions SRR1039770 and SRR1039771) as well as the corresponding RNA-Seq experiments (SRR1039764 and SRR1039765). The Activated Egg and Oocyte reads were concatenated to obtain Ribo-Seq and RNA-Seq “Egg” samples, which were

analysed employing the same pipeline as our own data. In the case of S2-cell data, we concatenated our previously-published Ribo-Seq (accessions SRR1548656, SRR1548657, SRR1548658, SRR1548659) and RNA-Seq datasets (SRR1548660-SRR1548661) [23]). We obtained modENCODE RNA-Seq embryonic-staged datasets from FlyBase and consolidated them into our timed collections (0h-8h, 8h-16h and 16h-24h AEL).

Comparison between RNA-seq and mass spectrometry datasets

We compared the mass spectrometry-based developmental proteome of *Drosophila melanogaster* from [26] with our own sequencing data by first averaging the imputed log2 LFQ intensity per protein-group across samples spanning embryonic windows matching those of our timed collections (0h-8h, 8h-16h and 16h-24h AEL). Finally, we normalized all datasets and measured the correlation between the average protein intensities for all identified proteins and our own gene expression measurements using Spearman´s rho.

Translation Efficiency and Translational Regulation analysis

Translation efficiency (TE) per ORF was calculated as the fraction of ribosome-density (Poly-Ribo-Seq RPKM) over the total mRNA read density (RNA-Seq RPKM). To detect significant events of translational regulation, we performed Z-ratio calculations of TE variation as per Cheadle et al. 2013 [46]. First, TE values were normalized by calculating the Z-score of each ORF in each sample:

$$Zscore_{ORF} = (TE_{ORF} - TE_{sampleMean}) / TE_{sampleSD})$$

Second,Z-ratios across contiguous developmental stages e.g. Early and Mid-embryogenesis were calculated for each ORF:

$$Zratio = (AverageZScore_{Mid} – Average\ Zscore_{Early)} / (ZScore_{Mid} – Zscore_{Early})_{SD}$$

Third, the cut-off of ≥|1.5|was used to identify biologically significant differences [45, 46] across the two stages.

Gene Expression enrichment analysis

We used the unique gene identifiers (FBgn) for genes identified as translationally-regulated across stages (see Z-ratio analysis) to calculate the embryonic tissue-

expression enrichment using the Intermine tool (Flymine), which uses ImaGO terms associated with expression patterns deposited in the BDGP database. The enrichment for each gene set was measured against a background of all *Drosophila* melanogaster genes with expression information, and expressed as a p-value for each significantly-enriched tissue, after the Benjamini-Hochberg multiple-comparisons correction.

Conservation analysis

The pre-computed phyloP nucleotide scores for 27-way insect multiple sequence alignments were downloaded from UCSC as bigWig files. We then used the UCSC bigWigAverageOverBed package to compare phyloP scores with BED files containing genome-coordinates for each ORF in our set, obtaining average ORF phyloP scores.

Amino acid composition analysis

We used a previously published own script (*aa_composition.pl*, [23]) to calculate the observed or predicted amino acid compositions of our different sets of ORFs.

Kozak-context scoring per ORF

For all canonical ORFs, we extracted the nucleotide composition around-but excluding-the annotated START codon (-5 to 6, excluding positions 1-3). For each position, we then calculated the log odds ratio between observed and background nucleotide frequencies. This provided a scoring table of position-specific nucleotide frequencies from *bona-fide* Kozak contexts with which to score individual ORFs in all classes. The final score per ORF was then obtained by adding the individual position-specific values for all observed nucleotides.

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Figure 1 - Experimental design and quality-control of Poly-Ribo-Seq data. A *Drosophila melanogaster* embryos were collected in three contiguous time-windows spanning the whole of embryogenesis. Two biological replicates were collected for each window, for both Poly-Ribo-Seq and RNA-Seq. pro: procephalon; gb: germ band; df: germ band; mg: presumptive midgut **B** Percentage of 5'UTR, 3'UTR and CDS-aligned reads for Poly-Ribo-Seq, across the annotated transcriptome of *Drosophila melanogaster*. **C** Reproducibility of RPKM values for canonical ORFs by Spearman correlation (*r*) across replicates T (x-axis) and B (y-axis), per stage, for both Poly-Ribo-Seq (top panels) and RNA-Seq (bottom panels). **D** Density of genome-aligned reads for all experiments, both Poly-Ribo-Seq and RNA-Seq across the polycistronic *tarsal-less* (*tal*) transcript.

Figure 2 – Detection of Translation in annotated and non-annotated ORFs. A Sequentially inclusive pipeline used for translation calls. An ORF was considered translated, in each stage, if it showed RPKM > 1 in one of two RNA-Seq replicates, RPKM > 1 in both Poly-Ribo-Seq replicates and a *p*-value < 0.01 in the binomial test for framed-reads. **B** Metagene plot for codon framing of 32-nt. ribosome footprints across all ORFs analysed in this study, showing three-nucleotide periodicity (framing) in the third position of each codon (frame 2, blue). Numbers on top denote the distances between the 5'-end of ribosomal footprints and START/STOP codons. **C** The observed framing for 32-nt. Ribosome footprints is consistent across embryonic stages (combined replicates per stage). **D** Logic of per-ORF analysis of framing probabilities using the binomial test. **E** Density of 32 nt. genome-aligned Poly-Ribo-Seq reads for all experiments, per frame, and corresponding translation probability for the *scl-A* ORF, as measured by the binomial test. **F** Numbers of developmentally-transcribed, ribosome-bound and translated canonical ORFs (annotated ORFs with length > 100 codons, shortCDSs (annotated ORFs ≤ 100 codons) and uORFs.

Figure 3 - Comparisons across genome-wide datasets. A Per-stage Spearman´s correlations across Poly-Ribo-Seq ("FP"), RNA-Seq ("mRNA") and mass-spectrometry datasets ("E"-Early, "M"-Mid, "L"-Late stages correspondence in *Casas-Vila* et al. 2017 - see Methods). "Maternal" datasets correspond to concatenated Mature Oocyte and Activated Egg datasets from *Kronja et al. 2014*. S2-cells correspond to the *Aspden et al. 2014* dataset plus of novel sequencing. Numbers denote Spearman's *rho*. **B** Numbers and translation fates of Maternal ribosome-bound canonical ORFs in the maternal-to-zygotic transition. "Constant translation" denotes ORFs detected as translated by our pipeline in both Maternal and Early embryo datasets (see Figure 2A). "Poised for translation" denotes ORFs showing Maternal ribosome-binding which only appear as translated in the Early embryo. "Downregulated translation" denotes ORFs with Ribosome-binding and translation in the Maternal dataset, which do not appear in the Early embryo dataset. **C** Overall correlation between average embryo Poly-Ribo-Seq RPKM and average embryo Mass-Spec imputed LFQ intensities (both datasets are log_2-transformed). All detected ORFs are included in this analysis. **D** Differential detectability of canonical ORFs between Poly-Ribo-Seq and Mass-spectrometry techniques. **E** Differential detectability of shortCDSs between Poly-Ribo-Seq and Mass-spectrometry. **F** S2-tagging experiments of uncharacterized shortCDSs showing translation.

Figure 4 - Regulated translation during embryogenesis. A Number of transcribed, ribosome-bound and translated ORFs per class across *Drosophila melanogaster* embryonic development, showing progressive specificity in ON/OFF translation patterns. **B** Changes in average translation efficiency, per ORFs class, across analysed developmental time-points. **C** Z-score ratio analysis of translation efficiencies (TE) during successive developmental stages pinpointing ORFs subjected to significant developmental modulations of translation across ORF classes. **D** Number of developmentally-regulated canonical ORFs subjected to downregulation (bottom, Z-ratio ≤ -1.5) or upregulation (top, Z-ratio ≥ 1.5), per developmental transition. **E-F** Significantly-enriched gene expression patterns in the translationally-upregulated canonical genes the Early-to-Mid and Maternal-to-Early developmental transitions respectively (*p-value* of BGDP expression annotations after Benjamini-Hochberg procedure).

Figure 5 - uORFs as translational regulators. A Variation in canonical TE levels as a function of the number of cistronic uORFs showing ribosomal binding. **B** Comparison of translational changes between uORFs and cognate canonical ORFs across developmental stages. Numbers denote the number of Z-ratio significant uORF-mORF pairs in each quadrant. Vertical arrows: uORF translational regulation sign. Horizontal arrows: cognate canonical translational regulation sign. Green arrows: Upregulated; Red arrows: Downregulated. **C** Correlation between the number of uORFs per mRNA and 5'UTR length (Spearman's r = 0.7). **D** Comparison of Kozak-context scores between distinct ORF classes and uORF subclasses. Graphs display 10-90 percentile range. Mann-Whitney $p < 0.001$. **E** Comparison of phyloP conservation scores (27-way alignments) between distinct ORF classes and uORF subclasses. Graphs display 10-90 percentile range. Mann-Whitney $p < 0.01$. **F** Correlations in amino acid usage across distinct uORF subclasses, shortCDSs and canonical ORFs; values denote pairwise Spearman's r. **G** Tagged uORFs showing translation in S2 cells. **H** Tagged dicistronic ORFs showing translation in S2 cells. **I** uORF evolution model. 5'-leaders in blue. t: evolutionary time. Red lines denote ribosomal binding; white boxes correspond to novel uORFs; salmon boxes correspond to ribosomal-bound uORFs; red boxes correspond to translated uORFs.

Supplementary Figure 1 – RiboSeq read length distribution and transcriptome-wide framing. A Distribution of Poly-Ribo-Seq genome-mapped ribosome-protected fragment (RPFs) lengths; codon-framing of predominant lengths shown in inset. **B** negative control: of codon-framing in genome-mapped RNA-Seq reads fails to show dominance in any of the frames.

Supplementary Figure 2 – S2-cell canonical translation. A Comparison of translated canonical ORFs in S2 cells and the late embryo stages from which these cell culture primarily originated (Schneider). **B** Comparison of ribosome-bound and translated canonical ORFs in S2 cells, showing that most Ribosome-binding produced measurable translation.

Supplementary Figure 3 – uORF A Comparison of uORF translation from T and B replicas (in $\log_2$RPKM) show a very high correlation (Spearman's *r*=0.9466). **B** Histogram of individual Spearman's correlation *r* between TE of ribosome-bound uORFs and their corresponding mORF across embryogenesis. The overall positive correlation has a median of *r*=0.477. **C**. Distribution of $RPKM^{FP}$ log2 values of ribosome-bound-only uORFs (red) and translated (framed) uORFs (green).

Supplementary Table 1 – Sequencing statistics for novel RiboSeq and RNA-Seq datasets reported in this study. Number of raw reads per replica per stage and percentage of remaining reads after ribosomal RNA subtraction (norRNA) and genome-mapping (see methods).

Supplementary Table 2 – Heatmap of correlations across RNA-Seq and RiboSeq samples and replicates Spearman correlation values (*r*) for canonical ORF RPKMs across all RNA-Seq and RiboSeq replicates analysed in this study. All correlations were significant (p<0.0001). T and B denote the two biological replicates used in this study. Early, Mid and Late denote Embryonic windows 0-8h, 8-16h and 16-24h, respectively. "Flybase" RNA-Seq refers to modENCODE data (Graveley et al. 2011). S2 refers to S2-cell datasets (see methods). Unfertilized Egg corresponds to previously-published data (Kronja et al. 2014).

Supplementary Tables 3 - Gene expression, regulation and conservation statistics for distinct small ORFs in polycistronic mRNAs. A The *tal* polycistronic mRNA shows marked quantitative differences in translation efficiency, probability and regulation, as well as conservation, across the four ORFs. **B** The *scl* locus contains two cistronic ORFs that show similar but quantitatively different levels of translation and conservation.

Figure 1

Method and basic data

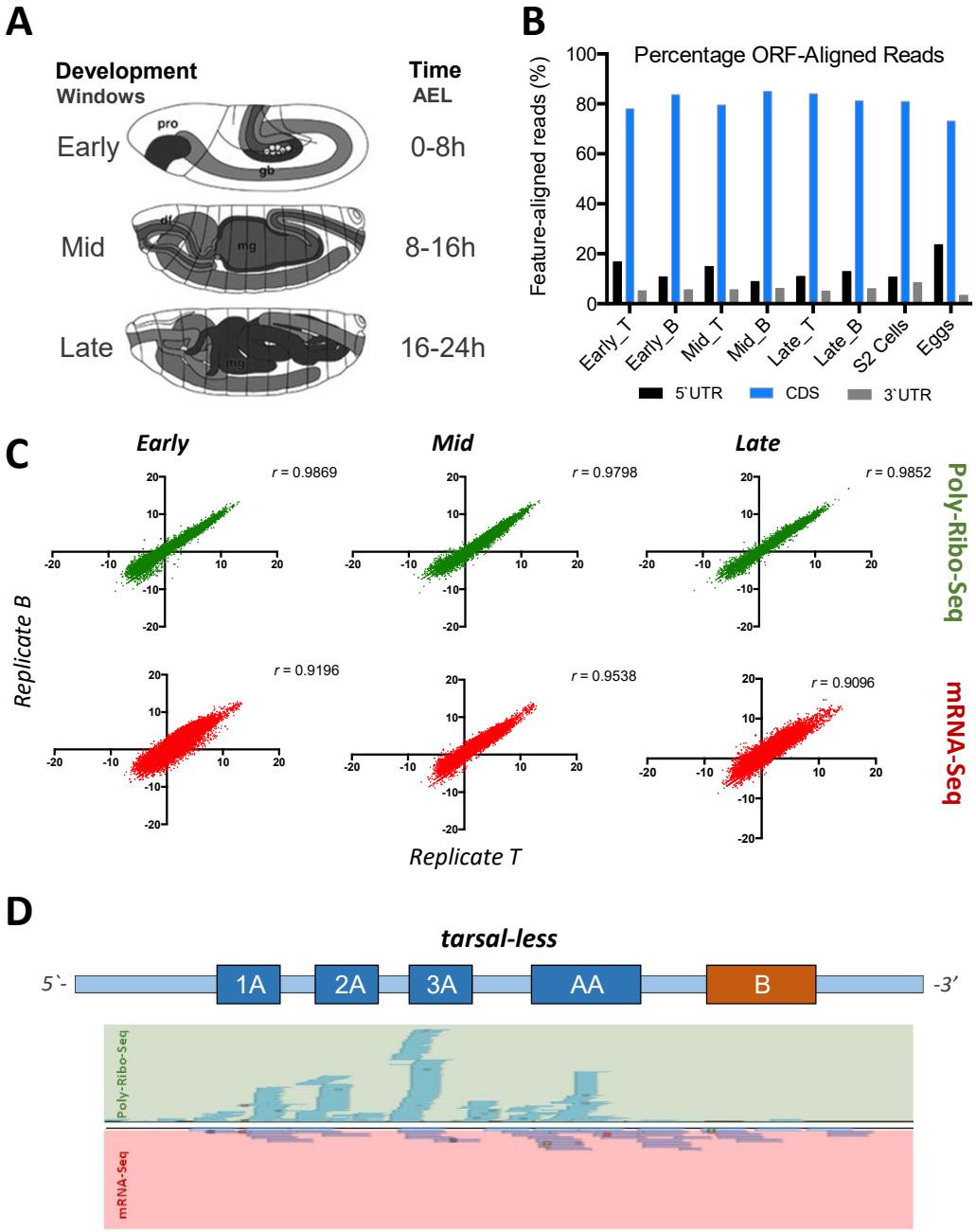


Figure 2

Detecting Translation

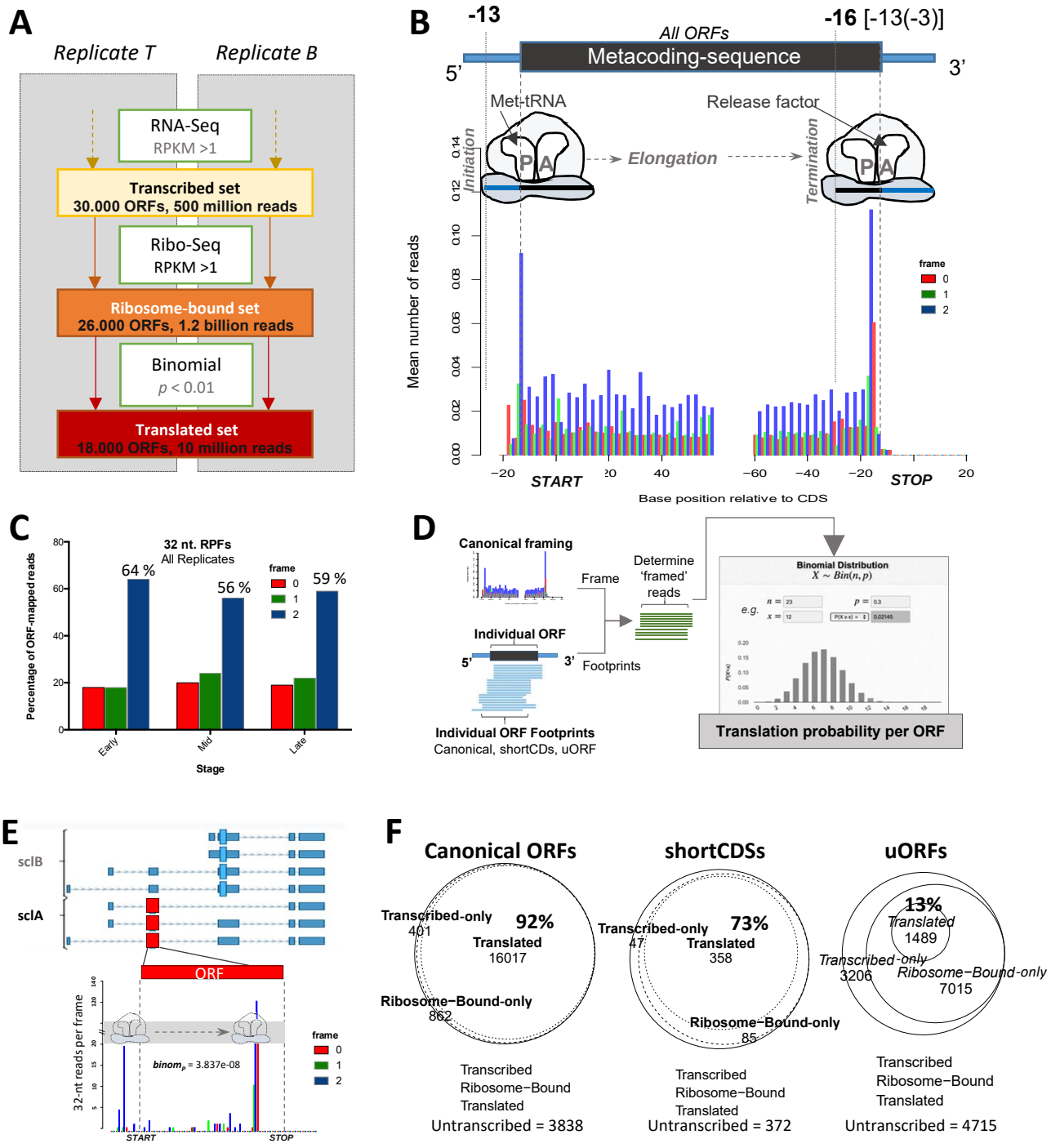


Figure 3

Comparison among genomic data

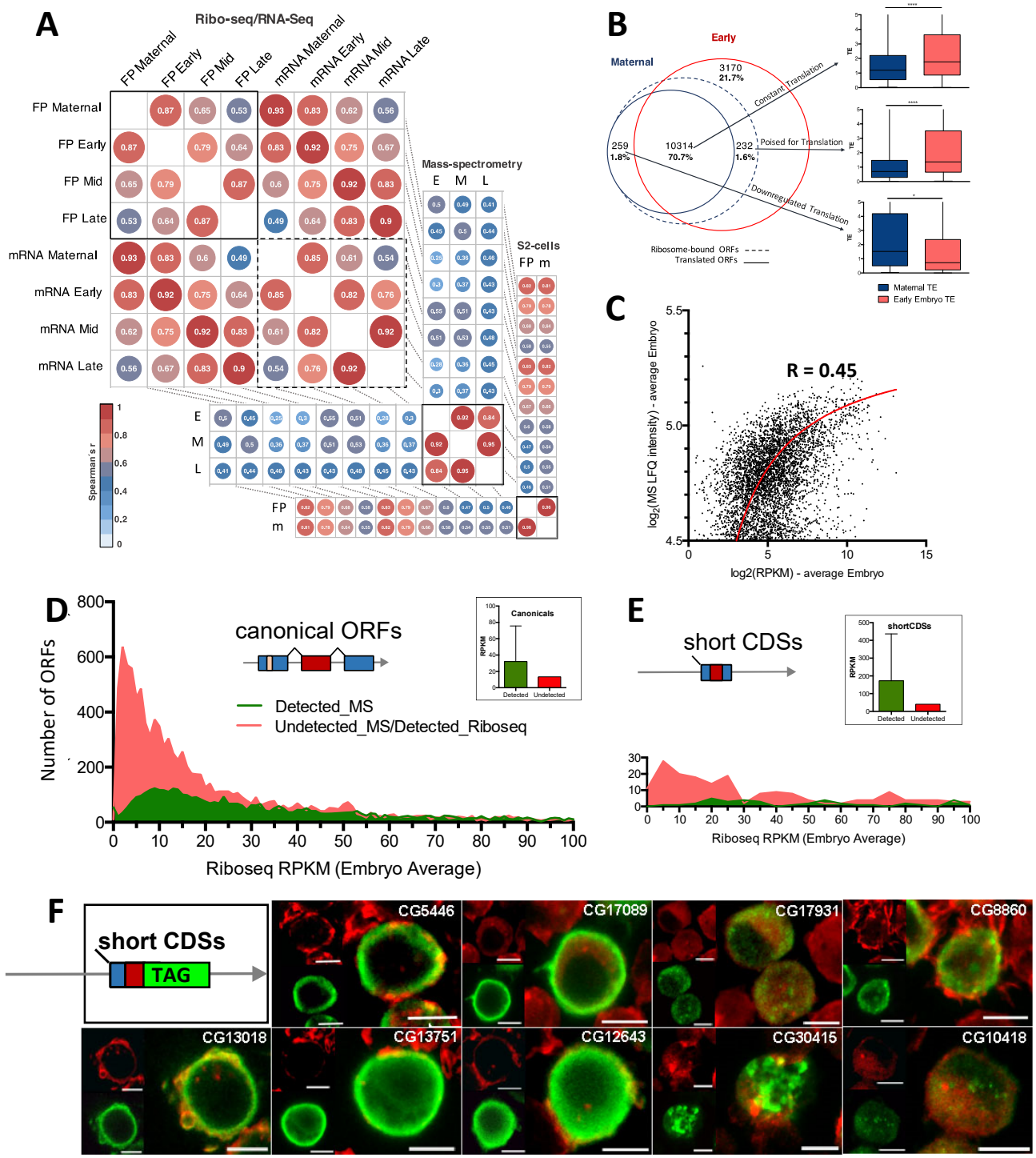
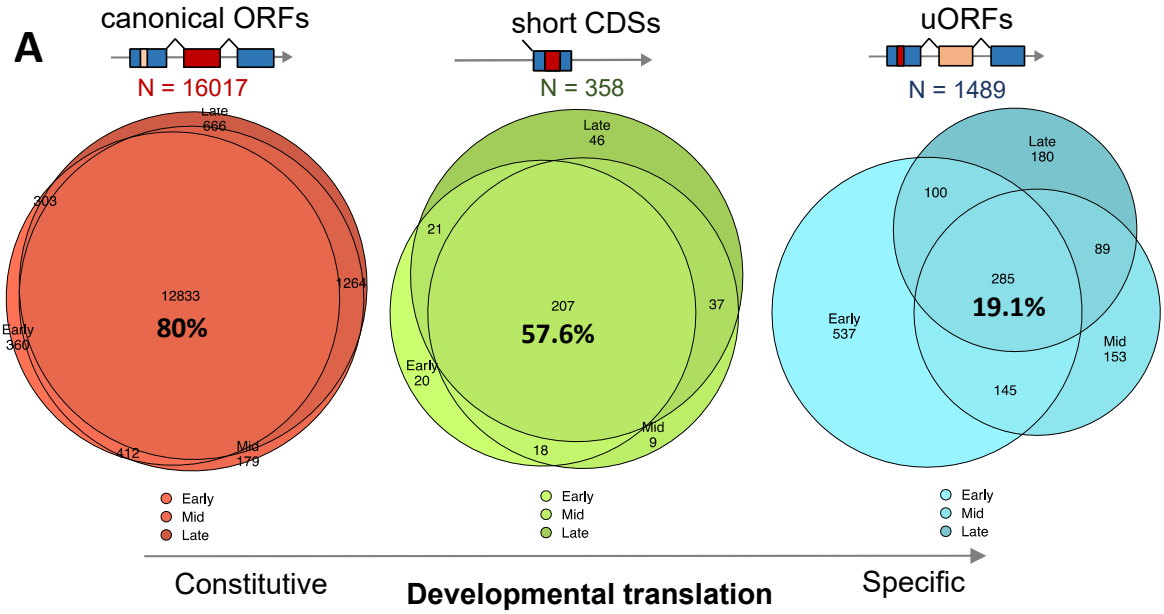
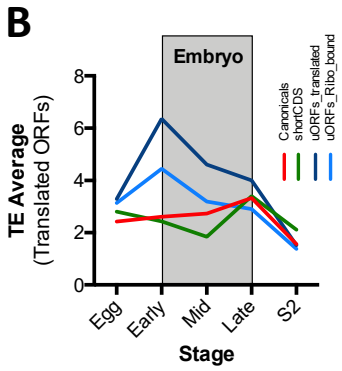


Figure 4

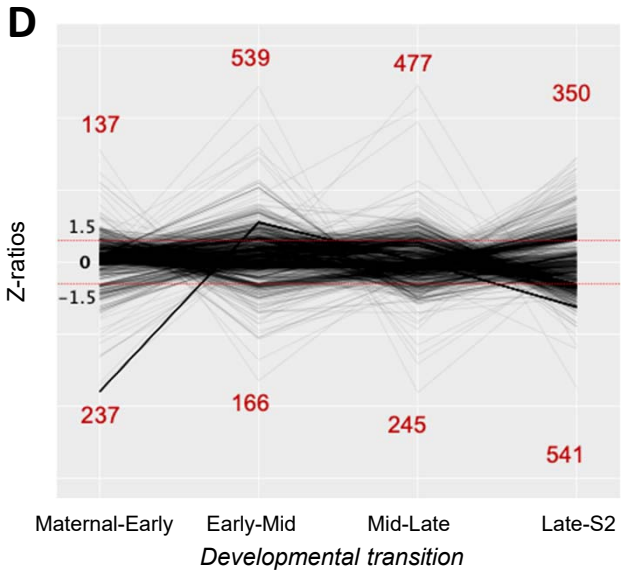
Regulated translation during embryogenesis





C

			Translationally-regulated			
Class	**Translated**	**Maternal-to-Early**	**Early-to-Mid**	**Mid-to-Late**	**Late-to-S2**	**Total**
Canonicals	16017	374	705	722	891	1904 (**12 %**)
Short CDSs	358	6	20	29	20	57 (**16%**)
$uORFs_{translated}$	1489	78	147	130	59	270 (**18 %**)
$uORFs_{ribo-bound}$	7015	217	350	334	186	767 (**10%**)



E

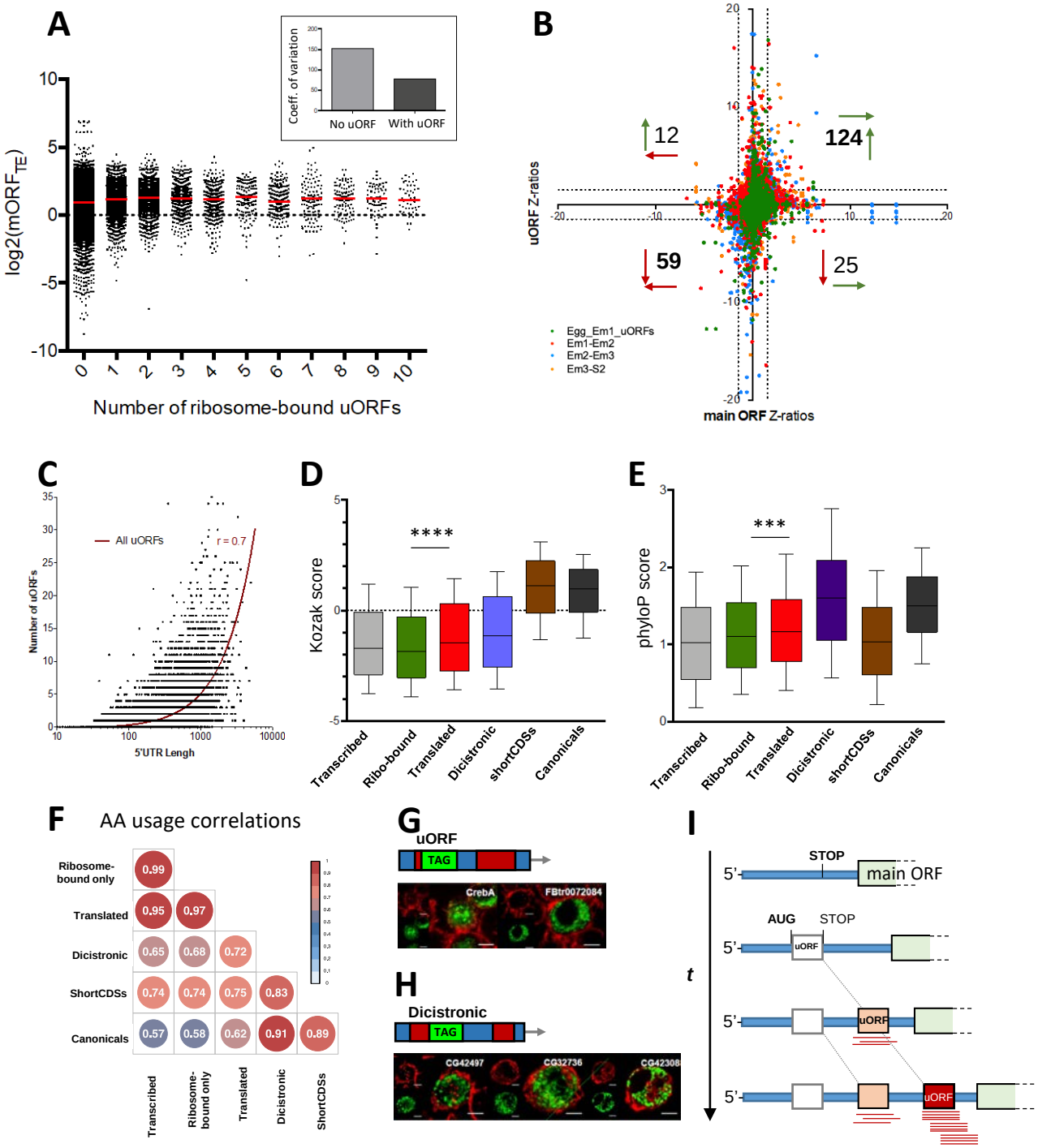
228 genes (539 ORFs) – Up Early-to-Mid

Terms	p-Value	Matches
ventral nerve cord	6.345764e-4	52
embryonic dorsal epidermis	0.001006	35
embryonic brain	0.002130	49
embryonic epipharynx	0.002235	20
embryonic hypopharynx	0.003883	20
embryonic foregut	0.005816	22
embryonic esophagus	0.005891	14
embryonic ventral epidermis	0.008728	29
embryonic/larval tracheal system	0.011728	17

F

100 genes (161 ORFs)– Up Maternal-to-Early

Terms	p-Value	Matches
ubiquitous	0.003625	35
maternal	0.038710	49

Figure 5

uORFs as translational regulators