

## Single molecule imaging and modelling of mRNA decay dynamics in the *Drosophila* embryo

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Short title: mRNA dynamics and degradation in the *Drosophila* embryo

## 1 **Abstract**

2 Regulation of mRNA degradation is critical for a diverse array of cellular processes and developmental  
3 cell fate decisions. Many methods for determining mRNA half-lives rely on transcriptional inhibition or  
4 metabolic labelling. Here we use a non-invasive method for estimating half-lives for hundreds of mRNAs  
5 in the early *Drosophila* embryo. This approach uses the intronic and exonic reads from a total RNA-seq  
6 time series and Gaussian process regression to model the dynamics of premature and mature mRNAs.  
7 We show how regulation of mRNA stability is used to establish a range of mature mRNA dynamics  
8 during embryogenesis, despite shared transcription profiles. Using single molecule imaging we provide  
9 evidence that, for the mRNAs tested, there is a correlation between short half-life and mRNA association  
10 with P-bodies. Moreover, we detect an enrichment of mRNA 3' ends in P-bodies in the early embryo,  
11 consistent with 5' to 3' degradation occurring in P-bodies for at least a subset of mRNAs. We discuss  
12 our findings in relation to recently published data suggesting that the primary function of P-bodies in  
13 other biological contexts is mRNA storage.

14

## 15 **Introduction**

16 Cells establish their identity by changing their gene expression patterns in response to different signals  
17 and environments. Critical to this is the ability of a cell to modulate mRNA levels. mRNA abundance  
18 depends not only on the transcription rate but also on mRNA stability. In eukaryotic cells, there are two  
19 major pathways of mRNA degradation: Xrn1 endonuclease-mediated 5'-3' decay and exosome  
20 catalysed 3'-5' decay [1,2]. Many mRNA degradation factors and mRNAs can become condensed into  
21 processing bodies (P-bodies), which are phase separated compartments in the cytoplasm implicated in  
22 mRNA storage and decay [3,4]. mRNA stability is also commonly regulated by sequences in the 3' UTR,  
23 including binding sites for RNA binding proteins or miRNAs [5]. While the two major decay pathways  
24 are responsible for general turnover of cytoplasmic mRNAs, there are also mRNA surveillance pathways  
25 that degrade aberrant mRNAs. These include mRNAs carrying a premature stop codon, lacking a stop  
26 codon, or mRNAs with paused ribosomes [6].

27 Regulation of mRNA degradation is essential for diverse cellular processes including  
28 proliferation, differentiation, apoptosis and immune responses [1,7–10]. Control of mRNA stability is also  
29 important for cellular decisions and behaviour during development. For example, regulation of *myc*  
30 mRNA stability fine-tunes the proliferation rate of neuroblasts in the *Drosophila* larval brain [11], an *fgf8*  
31 mRNA gradient generated by mRNA decay couples differentiation to posterior elongation of the  
32 vertebrate embryonic axis [12] and *Hes1* mRNA instability is integral to the *Hes1* protein ultradian  
33 oscillations that may act as a timer for vertebrate neuronal differentiation [13]. In addition, a conserved  
34 feature of early embryogenesis is that there is bulk degradation of maternal mRNAs around the time of

35 zygotic genome activation [14,15]. Consistent with the key roles of mRNA stability in cell biology,  
36 mutations in many components of the degradation pathways are associated with human diseases [9,16].

37        While the half-lives of strictly maternal mRNAs during embryogenesis can be readily measured  
38 genome-wide [17], measuring the decay of zygotic mRNAs is more difficult due to ongoing transcription.  
39 One approach is to inhibit transcription and then follow the decline in mRNA levels over time [18–20].  
40 Other methods involve metabolic labelling of the RNA, for example in pulse-chase or approach-to-  
41 equilibrium experiments [19–21]. Related approaches use computational models to estimate  
42 transcription and degradation rates by sequencing both the total and labelled RNA following the pulse  
43 [19]. Single molecule fluorescent in situ hybridisation (smFISH) imaging based methods for estimating  
44 mRNA half-lives have also been described. However, these methods are not high throughput and  
45 require either steady-state transcript levels [22] or a natural shut off of transcription [23].

46        In this study we generate a high-resolution total RNA-seq time series across early  
47 embryogenesis that we use to estimate half-lives and assign mRNAs into different stability classes. Our  
48 data suggest that some mRNAs can be degraded in P-bodies, as the unstable mRNAs we have  
49 investigated are more highly colocalised with P-bodies and we can detect 3' mRNA fragments in P-  
50 bodies. Overall, our data reveal the contribution of mRNA stability to shaping mRNA levels during early  
51 embryogenesis and provide insight into how mRNA stability is regulated.

52

53

## 54 **Results**

### 55 **Isolation of mRNA from early embryos captures high resolution transcriptional dynamics**

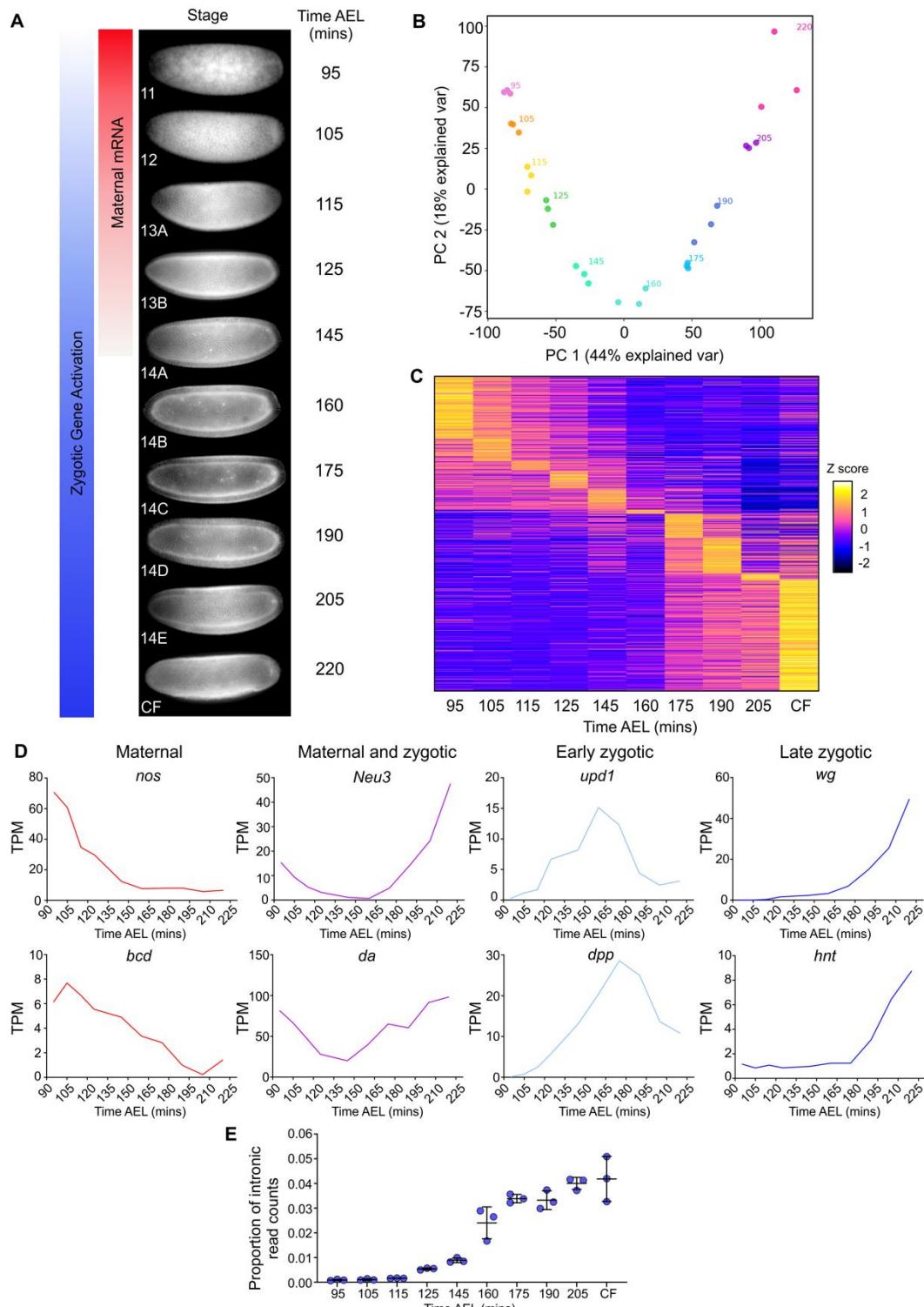
56 To investigate mRNA accumulation dynamics during early *Drosophila* embryogenesis, we first  
57 generated a total RNA-seq time series. The early *Drosophila* embryo undergoes a series of 14 nuclear  
58 cycles within a common cytoplasm (nc1-14). RNA was isolated from single early *Drosophila* embryos at  
59 10 time points, starting at nc11, ~ 90 minutes after egg lay (AEL) and prior to the onset of bulk zygotic  
60 transcription through to the beginning of gastrulation (Figure 1A). Embryos were collected from a  
61 His2avRFP transgenic line and precisely staged at nc11, nc12, nc13 and nc14 by calculating an  
62 internuclear distance ratio (Figure S1A). Single embryos were collected in triplicate 5 min after the nc11  
63 and nc12 divisions, both 5 and 15 min after the nc13 division, then at 15 min intervals during the long  
64 nc14 interval, with the final time point corresponding to the appearance of the cephalic furrow (Figure  
65 1A, Table 1). As male and female embryos have differences in X chromosome transcription due to  
66 dosage compensation [24], we used PCR to determine the sex of each embryo and select female  
67 embryos for analysis. We sequenced total RNA following rRNA depletion, rather than selecting for  
68 polyadenylated RNA, allowing us to capture intronic reads and other non-coding RNA species. The  
69 intronic reads allow quantification of nascent, unspliced transcripts and also detection of early zygotic

70 expression by distinguishing zygotic transcripts containing introns from maternally loaded spliced  
71 mRNAs.

72 We detected a total of 18159 transcripts during early embryogenesis representing 9026 unique  
73 genes. Using principal component analysis (PCA) we observed that the first two principal components  
74 represented 44% and 18% of the variation respectively and the replicates at each time point clustered  
75 together (Figure 1B). This suggests the biological age of the embryos explains the majority of variation  
76 within the data rather than differences between replicates, indicating the high quality of the libraries.  
77 Transcript levels across embryogenesis were visualised as a heatmap, with the transcripts ordered  
78 based on the time point of peak expression (Figure 1C). We classified 4897 early peaking transcripts  
79 (at the 95 or 105 min time points) as maternal, and 13262 transcripts peaking after 105 min as zygotic.  
80 Of the zygotic transcripts, 23% show peak expression early in nc13 or the start of nc14 (between 115  
81 and 160 min inclusive) and the remainder show late peak expression after 160 min. Analysis of different  
82 dynamically expressed genes showed that our dataset included well characterised maternal (*nos* and  
83 *bcd*), maternal and zygotic (*Neu3* and *da*), early zygotic (*upd1* and *dpp*) and late zygotic mRNAs (*wg*  
84 and *hnt*) (Figure 1D).

85 As we sequenced total RNA, we determined the number of reads that mapped to introns as well  
86 as exons and transcripts. Analysis of the distribution of intronic reads shows an even read coverage  
87 across introns over all time points (Figure S2A). Only a very small proportion of transcripts at time points  
88 105-125 min have intronic reads (Figure 1E), suggesting there is only minor zygotic transcription of  
89 intron-containing genes during these early stages. Previous studies have shown that the earliest zygotic  
90 activation of the *Drosophila* genome is biased towards expression of short intronless genes [25,26],  
91 which we cannot distinguish from maternally deposited transcripts at the early time points in our data.  
92 In addition, the early nuclear cycles are short, limiting the time period of active transcription.  
93 Nonetheless, eight genes have detectable levels of intron signal at nc12 and nc13A, suggesting early  
94 zygotic transcription (Figure S2C-D).

95



96

97 **Figure 1. Total RNA-seq captures dynamic gene expression during early *Drosophila***  
98 **development.** (A) The time points used to collect RNA-seq samples, with approximate times after egg  
99 lay (AEL) at 20°C, are shown ranging from early nc11 through to cephalic furrow (CF) formation. The  
100 schematic highlights how the embryo switches from relying on maternally loaded RNAs (red) to  
101 activating its own zygotic transcription (blue). (B) Principal component plot of the RNA-seq samples  
102 shows tightly correlated replicates at each time point. (C) A heatmap of all expressed transcripts with

103 TPM >1 (18159 transcripts). Data are Z normalised and ordered along the y axis by the time point of  
104 peak expression. (D) Different examples of transcript dynamics captured by the data, as indicated by  
105 the graph titles. (E) Proportion of normalised intron reads (RPKM) throughout the time series.

106  
107 The proportion of intronic reads increases significantly at 125 min (Figure S2D), then there is a  
108 further large increase around mid-nc14 (Figure 1E), when bulk activation of zygotic transcription occurs  
109 [24]. We detect 7276 zygotically expressed genes, similar to a previous estimate based on GRO-seq  
110 data [27]. The benefit of the high temporal resolution of our data can be seen in examples of transient  
111 gene expression, such as the gene *runt* (*run*) which is expressed at nc12 then peaks in early nc13  
112 (Figure S2D). *run* has essential roles in patterning and transcriptional control of sex determination in  
113 early development, so the precise temporal regulation of its expression is likely to be important for these  
114 functions [28]. Additionally, we observe temporal changes in mRNA isoforms during development,  
115 exemplified by the genes *Meltrin* and *thickveins* (*tkv*) (Figure S3A and B). Their isoforms have altered  
116 coding sequences, which for the zygotically expressed isoform of the BMP receptor *Tkv* results in a  
117 shorter extracellular ligand-binding domain. We also detected expression of non-coding RNA species,  
118 such as those in the bithorax complex (Figure S3C). Overall, due to the high temporal resolution of our  
119 data and the ability to detect non-coding RNAs, we have a high quality dataset to investigate  
120 transcriptional dynamics in early *Drosophila* development.

121  
122 **Gaussian process regression provides estimates of transcript half-lives in early embryogenesis**  
123 As the degradation of maternal transcripts has been studied previously [17], we focused on the kinetics  
124 by which zygotic mRNAs are cleared in the early embryo. We used the intronic reads in our total RNA-  
125 seq dataset to represent pre-mRNA levels as a proxy for the transcription rate, while exonic reads reflect  
126 mature mRNA levels (Figure 2A). The intronic reads are correlated with NET-seq data from early  
127 embryos ( $\rho = 0.46, p = 5.1 \times 10^{-11}$ ) (Figure S2B), consistent with the intronic signal reflecting transcription  
128 dynamics. We assume that introns are co-transcriptionally spliced and rapidly degraded. In support of  
129 this, NET-seq data suggests that >95% of splicing events are co-transcriptional in the *Drosophila*  
130 embryo [29] and modelling of metabolic labelling and sequencing data from *Drosophila* S2 cells revealed  
131 that the median half-life of introns is 2 min [30].

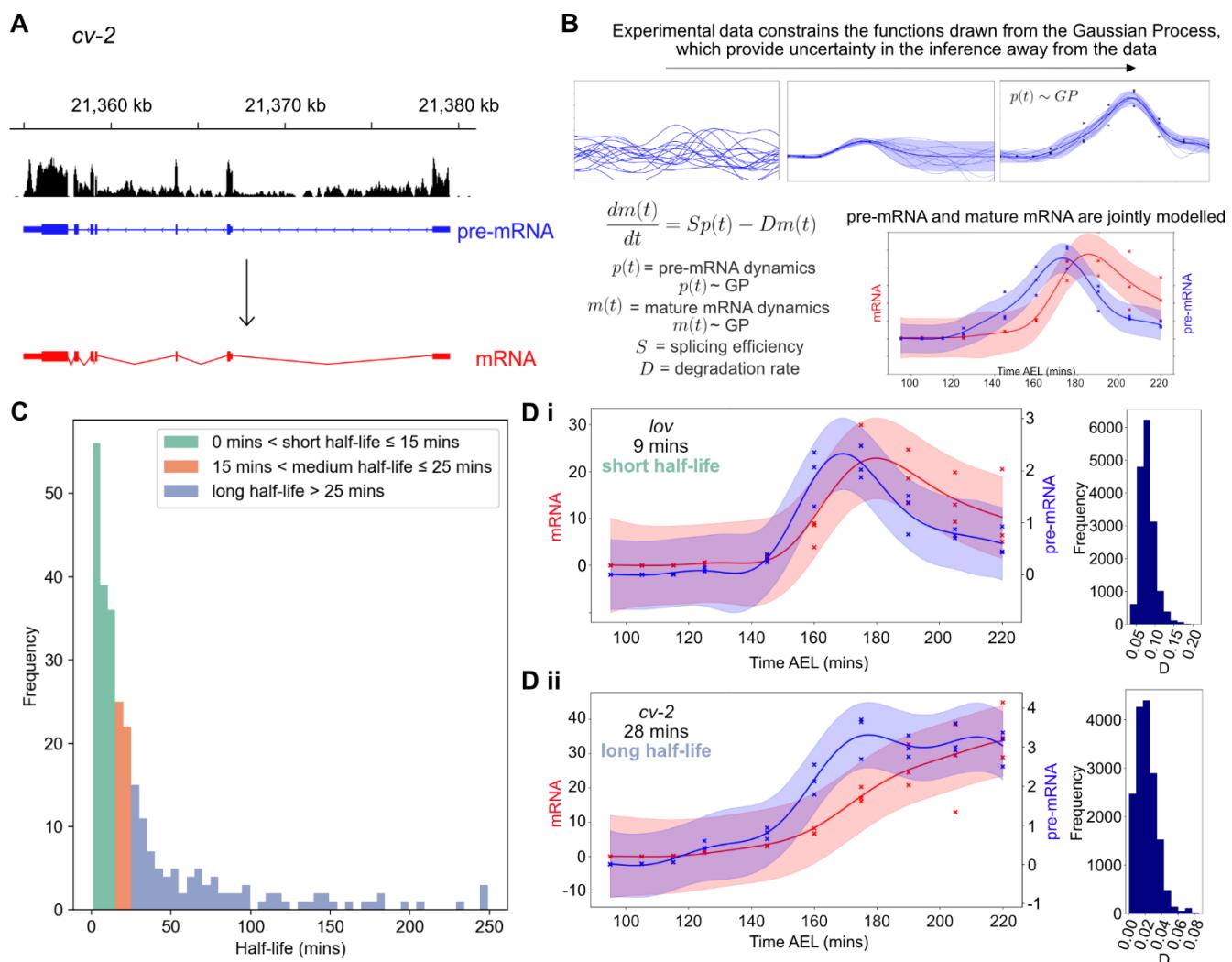
132 We used a Gaussian process (GP) regression model [31,32] of mRNA accumulation and  
133 degradation to estimate zygotic transcript half-lives from the intronic and transcript expression RNA-seq  
134 data (Figure 2B). Before fitting the GP regression, we applied a dynamic filter where we computed a  
135 log-likelihood ratio test between two GP regression models: a dynamic model with a radial basis function  
136 (RBF) kernel and a noise model to obtain genes that are differentially expressed. We then strictly filtered  
137 the dynamic data to select 593 mRNAs, which are purely zygotically transcribed and have very low  
138 reads at the first time point (<0.5 TPM). From these, we filtered further to select transcripts with a

139 correlation between the mRNA and pre-mRNA above 0.4. This is a mild correlation threshold that we  
140 applied to exclude transcripts for which pre-mRNA and mRNA are unrelated and it would therefore be  
141 hard to fit the model. As we filtered our data to select for zygotic genes we expect a positive correlation  
142 as gene introns and exons would increase together. The model uses a GP which specifies a prior  
143 distribution over possible underlying functions before observing the data. This non-parametric prior is  
144 governed by ordinary differential equations (ODEs), which describe the transcription regulation process.  
145 Once the data are observed, Bayesian inference is used to infer the posterior distribution. The posterior  
146 distribution allows quantifying uncertainty in the model as it reflects possible functions which can explain  
147 a given data set. Credible regions are derived from the posterior distribution to quantify the uncertainty  
148 at 95% confidence level. The ODE describing the system is shown in Figure 2B from which the splicing  
149 and degradation rates which are inferred using the GP regression. We assume that introns are spliced  
150 at the same rate for each mRNA, consistent with evidence from S2 cells that introns from the same  
151 mRNA tend to have similar splicing rates [30]. We normalised the read counts by the intron lengths so  
152 that the *S* parameter has comparable meaning for each mRNA, but allow it to differ from mRNA-to-  
153 mRNA to account for variation in splicing rates across genes [29] and also differences due to alternative  
154 splicing of different transcripts. For transcripts we used TPM units which normalises for transcript length.

155 The model provides half-life estimates for 263 zygotic transcripts corresponding to 186 genes  
156 (Supplementary Table 1). The distribution of these, coloured by short, medium or long half-life, can be  
157 seen in Figure 2C, with the mean half-life at 35 minutes and median at 16 minutes. Figure 2D shows  
158 examples of a gene with a short (Di) and a long (Dii) half-life, estimated using the GP model. Parameters  
159 were determined for these genes, along with associated uncertainty, using Markov chain Monte Carlo  
160 methods and the posterior distributions on the degradation rate *D* are displayed. *jim lovell (lov)* mRNA,  
161 a BMP target gene encoding a putative transcription factor [33,34], has a short half-life of 9 minutes  
162 whereas the *cv-2* mRNA, encoding a secreted protein that binds BMPs [35], has a longer half-life of 28  
163 minutes. Full parameter estimates and credible regions are shown in Figure S4A.

164 As the dynamic embryonic mRNAs are not at steady-state, a previously described smFISH  
165 based method developed in human cells [22] was unsuitable for validation of half-lives. An alternative  
166 method exploited the arrest of transcription during mitosis to calculate the *snail* mRNA half-life in the  
167 *Drosophila* embryo, based on quantitation of mRNA numbers before and after mitosis [23]. However,  
168 we found the variation between transcript numbers in different embryos to be greater than any reduction  
169 that would be expected over such a short time frame (~ 4 mins) due to degradation (Figure S4B-E). As  
170 a result, any reduction due to degradation is masked by high variation between embryos, as has  
171 previously been observed for other mRNA numbers in the *Drosophila* embryo [36]. The *snail* mRNA  
172 numbers are tightly controlled by negative autoregulation [23], suggesting that *snail* may be uniquely  
173 suited to this method for calculating half-life.

174 In the absence of direct half-life validation, we determined whether the types of factors encoded  
 175 by mRNAs with short and long half-lives have functions compatible with their inferred stabilities. Gene  
 176 ontology (GO) analysis reveals that, compared to all dynamic transcripts in the RNA-seq data, those  
 177 encoding transcription factors and cell adhesion proteins are enriched in the short ( $p$ -values  $2.3 \times 10^{-8}$   
 178 and  $4.1 \times 10^{-5}$ , respectively) but not long half-life mRNAs. Transcription factors have previously been  
 179 reported to be encoded by unstable mRNAs [17,37,38]. Therefore, this approach has allowed the  
 180 classification of transcripts into half-life categories where short half-life mRNAs are enriched for protein  
 181 functions reflected by their stability.  
 182

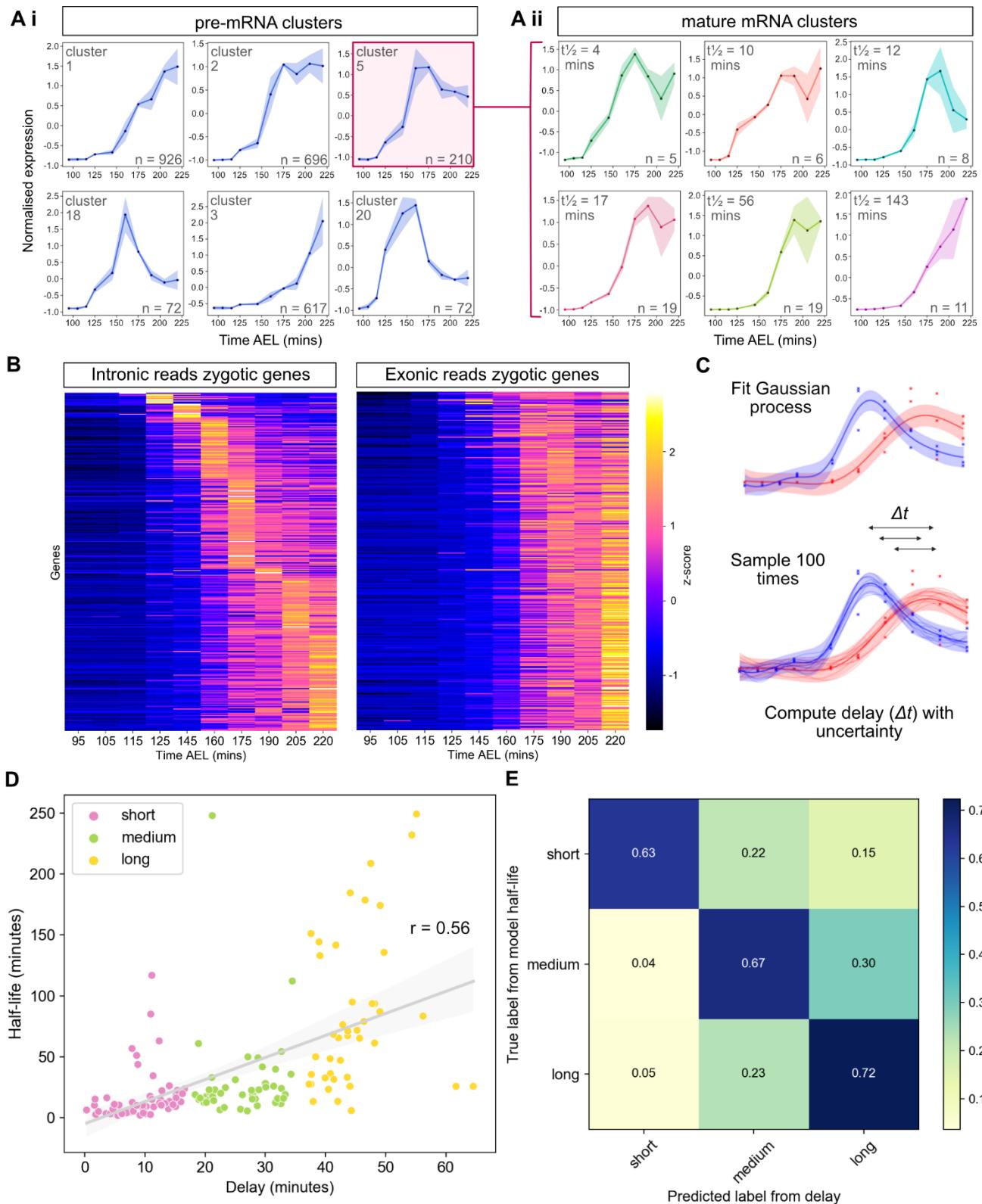


183  
 184 **Figure 2: Gaussian process regression provides estimates of transcript half-lives in early**  
 185 **embryogenesis.** (A) Reads aligned to intronic and exonic regions of genes are used to represent pre-  
 186 mRNA (blue) and mature mRNA levels (red), respectively. (B) Schematic of Gaussian Process  
 187 regression and the ODE model which shows the evolution of the mature mRNA dynamics is described  
 188 by the pre-mRNA data over time, sculpted by the splicing ( $S$ ) and degradation ( $D$ ) parameters. Pre-  
 189 mRNA and mature mRNA are therefore jointly modelled using GPs related by the ODE. (C) Half-life  
 190

191 results for 263 transcripts estimated using the GP model. Transcripts are divided into short, medium  
192 and long half-lives and coloured accordingly. (D) Examples of data for a short (i) and a long half-life  
193 mRNA (ii) *lov* and *pxb*, fit using the GP model. Pre-mRNA is shown in blue and mature mRNA in red,  
194 shaded areas represent credible regions and crosses mark the data for each experimental replicate at  
195 each time point. Posterior distributions for the degradation parameter  $D$  for each gene are shown to the  
196 right.  
197

## 198 **Clustering reveals how degradation shapes mature mRNA dynamics**

199 We next addressed how post-transcriptional regulation contributes to the range of mature mRNA  
200 dynamics seen in our data, by combining clustering analysis with our modelling of transcript half-lives.  
201 The pre-mRNA data were clustered using GPclust (Figure S5) [39]. From the intronic clusters, six highly  
202 populated intronic clusters that together exhibit a variety of interesting mRNA dynamics are shown in  
203 Figure 3Ai. The genes in each cluster share similar pre-mRNA profiles, and therefore transcription  
204 dynamics. All of the pre-mRNAs in intronic cluster 5 were then sub-clustered based on their mature-  
205 mRNA profiles (Figure 3Aii), which revealed that a range of mature mRNA dynamics arises from this  
206 single transcriptional profile. The zygotic mRNA subclusters for intronic cluster 2 also display a range of  
207 mature mRNA dynamics and are shown in Figure S6. The GP model sheds light on how these various  
208 dynamics arise, due to differences in the half-lives of transcripts in each cluster (Figure 3Aii, Figure S6).  
209 It is clear that the pattern in the shape of the time series is reflected in the different half-lives of the  
210 clusters; clusters which have a stronger peak have a shorter half-life and higher degradation rate,  
211 whereas those which continually increase across the time period have a long half-life and low  
212 degradation rate.



213

214 **Figure 3: Degradation regulates mRNA dynamics and can be approximated using the time delay**  
 215 **between peak pre-mRNA and mature mRNA expression.** (A) Clustering of pre-mRNA (Ai) and  
 216 mature (Aii) mRNA time series. Plots show the mean expression value at each time point for all mRNAs

217 in a cluster and the shaded area shows the standard deviation. The intronic cluster ID (Ai) or mean half-  
218 life (Aii) is shown in the top left and the number of transcripts in each cluster in the bottom right. (B)  
219 Heatmaps of pre-mRNA and mRNA expression at the gene-level for the subset of zygotic transcripts,  
220 ordered by the time point of maximum pre-mRNA expression. (C) Schematic illustrating the estimation  
221 of temporal delays between pre-mRNA and mature mRNA peak times ( $\Delta t$ , arrows) by fitting and  
222 sampling from a GP. (D) Correlation of the delay between the peak of pre-mRNA and mature mRNA  
223 against inferred half-life (Pearson's  $r = 0.56$ ,  $p = 6.5 \times 10^{-14}$  for testing non-correlation). Points  
224 representing transcripts are coloured by time delay category. (E) Confusion matrix comparing genes  
225 categorised into short, medium and long delays and their respective half-life categories. Numbers in the  
226 boxes indicate the fraction of genes with a given delay in the corresponding half-life category.  
227

228 As the clustering data indicated that half-life contributes to the shape of the mature mRNA profile,  
229 we further investigated the relationship between the relative timing of the peak of the pre-mRNA and  
230 mature mRNA. Visualisation of the gene-level pre-mRNA and mature mRNA data from the zygotic  
231 subset as heatmaps, reveals that for a given pre-mRNA peak time, there are a range of mature mRNA  
232 peak times with different delays (Figure 3B). Delay is defined as the time difference at which the peak  
233 is observed for the pre- and mature mRNA. The pre-mRNA and mature mRNA data were modelled  
234 using a Gaussian process which was then sampled with  $n = 100$ , so that the time delay between the  
235 peaks could be determined and the uncertainty in the estimate quantified (Figure 3C). The relationship  
236 between delay and half-life, for each transcript that has been modelled, is shown in Figure 3D. There is  
237 a moderately positive yet significant correlation between the two variables. Figure 3E shows the data  
238 as a confusion matrix in order to assess whether delay is predictive of half-life. Enrichment along the  
239 diagonal supports this; 63% of short delay genes have short half-lives; 67% of medium delay genes  
240 have medium half-lives and 72% of long delay genes have long half-lives. Together, these results reveal  
241 how post-transcriptional regulation is able to shape mature mRNA dynamics through regulation of  
242 mRNA half-lives and that the time delay between maximum expression of the pre-mRNA and mature  
243 mRNA can be used as an indicator of mRNA stability.

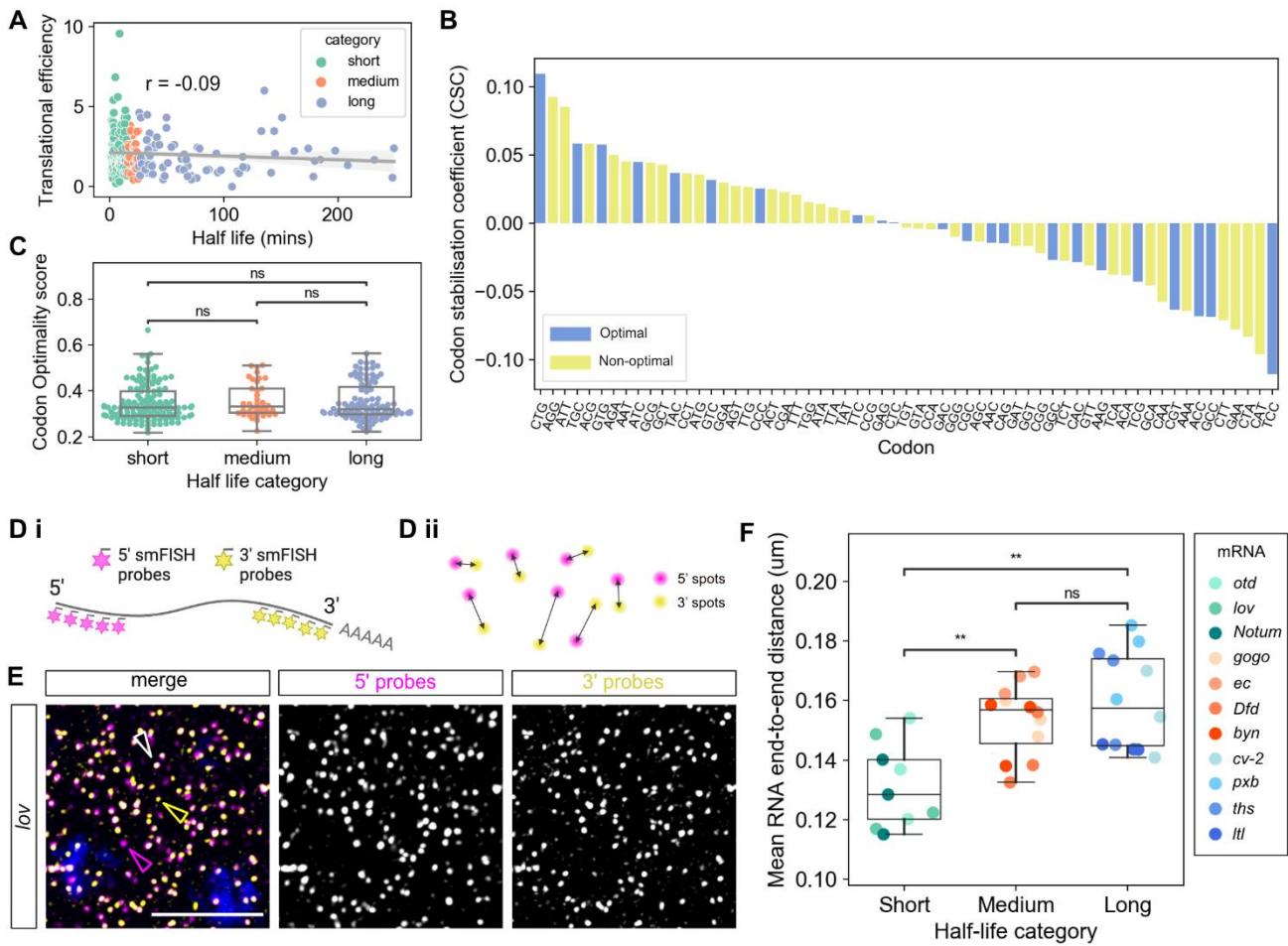
244

#### 245 **The short half-life mRNAs tested are more compact**

246 The degradation of an mRNA in the cytoplasm can be closely linked to its translation [1]. We therefore  
247 investigated how mRNA half-lives are shaped by both structural and sequence features known to  
248 influence translation. Regulatory sequences controlling mRNA degradation, translation and localisation  
249 are frequently located in the 3'UTR [40]. We found that 3'UTR length does not have any significant  
250 correlation with our inferred half-lives (Figure S7A), in agreement with previous studies of mRNA stability  
251 in late stage *Drosophila* embryos [37]. Similarly, there is no relationship between transcript length and  
252 stability in our dataset (Figure S7B).

253 Due to the links between mRNA decay, translation efficiency and codon optimality [41], we next  
254 investigated whether there is a relationship between half-life and both the translation efficiency and  
255 codon usage across the transcripts within our dataset. Using published ribosome footprint profiling data  
256 from 2-3 hour embryos [42] we plotted the translation efficiency and half-life for each of the transcripts  
257 within our dataset and observed no significant correlation between translation efficiency and half-life  
258 (Figure 4A). To extend this analysis, we also determined the codon stabilisation coefficient (CSC) for  
259 each codon which is a measure of the correlation between codon usage and stability of mRNAs. We  
260 plotted the CSC of each codon ordered by this value from highest to lowest (Figure 4B) and examined  
261 the identity of optimal codons previously defined in *Drosophila* embryos [43] and their occurrence within  
262 the CSC plot. The proportion of optimal codons is not significantly enriched within the positive and  
263 negative CSC groups (33% vs. 39%,  $p = 0.79$ , Figure 4B). There is also no significant difference in the  
264 proportion of optimal codons for transcripts within each of the different categories of half-life (Figure 4C)  
265 and clustering mRNAs based on codon usage showed that different clusters had similar half-lives  
266 (Figure S7C).

267 We next used imaging to analyse mRNA compaction in the context of stability. A more open  
268 conformation has been detected for specific mRNAs when they are being translated [44–46], raising the  
269 possibility that a particular conformation may also influence mRNA stability. We therefore selected a set  
270 of 11 zygotic mRNAs, 4 each from the medium and long half-life categories and 3 from the short half-  
271 life category (Figure S8A). A 4<sup>th</sup> short half-life mRNA, *Neu2*, is too short to separate the probe sets for  
272 compaction analysis but is included in further analysis (see later). We used dual-colour smFISH probes  
273 to visualise their 5' and 3' ends, and quantitate the distance between them, in fixed embryos (Figure  
274 4D). A representative smFISH image for one of the mRNAs, *lov*, is shown in Figure 4E, images for the  
275 other mRNAs tested are shown in Figure S8B.



276

277 **Figure 4: mRNA properties and stability.** (A) Half-life (x-axis) versus the translation efficiency from 2-  
278 3 hour embryos [42]. In all panels points representing transcripts are coloured by half-life category  
279 (Pearson's  $r = -0.09$ ,  $p = 0.18$ ). (B) Codon stabilisation coefficients calculated from our estimated half-  
280 lives showed no difference in the proportion of optimal (blue) and non-optimal codons (yellow) (chi-  
281 squared test  $p = 0.79$ ). (C) Proportion of optimal codons within transcripts from each half-life category.  
282 No significant difference was observed in the percentage of optimal codons within each category, tested  
283 by independent t-test (short vs med  $p = 0.5$ ; short vs long  $p = 0.7$ ; med vs long  $p = 0.7$ ). (Di) Schematic  
284 showing detection of the 5' (magenta) and 3' (yellow) ends of each mRNA with different smFISH probe  
285 sets. (Dii) Spots belonging to the same mRNA are matched (see Methods). (E) Maximum projection of  
286 6 slices from a confocal image showing smFISH detection of the 5' and 3' ends of *lov* mRNAs with lone  
287 5' ends, 3' ends and colocalised ends labelled by magenta, yellow and white arrowheads, respectively.  
288 Scale bars: 5  $\mu$ m. In the uncropped image from this embryo there are 5668, 1645 and 3620 intact, lone  
289 5' and lone 3' signals, respectively. For absolute numbers of intact mRNAs and lone ends for all mRNAs,  
290 see Figure S9A. (F) Graph shows the end-to-end distances of mRNAs with different stabilities,  $n = 3$   
291 embryos per mRNA. Data are mean distances across all colocalised mRNAs in each embryo ( $n > 220$   
292 whole RNAs for all images). Genes are grouped by their half-life category and the hue in each category  
293 corresponds to the order of the half-lives (lighter colour refers to shorter half-life). Short half-life mRNAs  
294 are more compact than both medium ( $p = 3.1 \times 10^{-3}$ ) and long ( $p = 1.6 \times 10^{-3}$ ) half-life mRNAs. No  
295 significant difference in end-to-end distance was seen between medium and long half-life transcripts ( $p$   
296  $= 8.9 \times 10^{-1}$ ).

297 For each image, the number and position of the 5' and 3' signals were collected and pairs were  
298 identified by solving a paired assignment problem (Figure 4Dii). For each pair, the distance between the  
299 5' and 3' signals was then measured; only ends with a distance less than 300 nm were assigned as  
300 same mRNA [46]. First, we estimated our smFISH detection efficiencies using alternating fluorophores  
301 for the *otd* and *lov* mRNAs (Figure S9Bi). These data reveal mean detection efficiencies of ~70% (Figure  
302 S9Bii), which is in the 70-90% range reported from other smFISH studies [47–51]. However, we note  
303 that our detection of the 670 labelled probe sets is generally slightly poorer than that of the 570 probes  
304 due to a lower signal to noise, consistent with findings from a previous study that used 670 labelled  
305 probes [49].

306 The distributions of end-to-end distances for each of the mRNAs tested reveal that short half-life  
307 mRNAs are significantly more compact, based on a smaller end-to-end distance, than mRNAs in the  
308 medium and long half-life categories (Figure 4F). Considering the lower detection limit of the imaging  
309 setup we used is ~120nm (see Methods), we found that for our alternating probe sets and the 5' and 3'  
310 compaction data, the *otd* and *lov* short half-life mRNAs had an end to end distance that is very compact  
311 and close to this limit (Figure 4F). No significant difference is observed in the end-to-end distance for  
312 mRNAs in the medium and long half-life categories however we did find some mRNAs in the long  
313 category were in a more open conformation than those in the medium category (Figure 4F). We also  
314 identified unpaired mRNA ends (see later), which were further apart than the 300 nm distance threshold  
315 used. Finally, quantitation of additional control smFISH experiments for some of the test mRNAs, in  
316 which the fluorophore dyes on each set of probes were switched (Figures S9C) in order to control for  
317 detection differences between the channels mentioned above, also revealed significantly shorter end-  
318 to-end distances for short half-life mRNAs (Figure S9D).

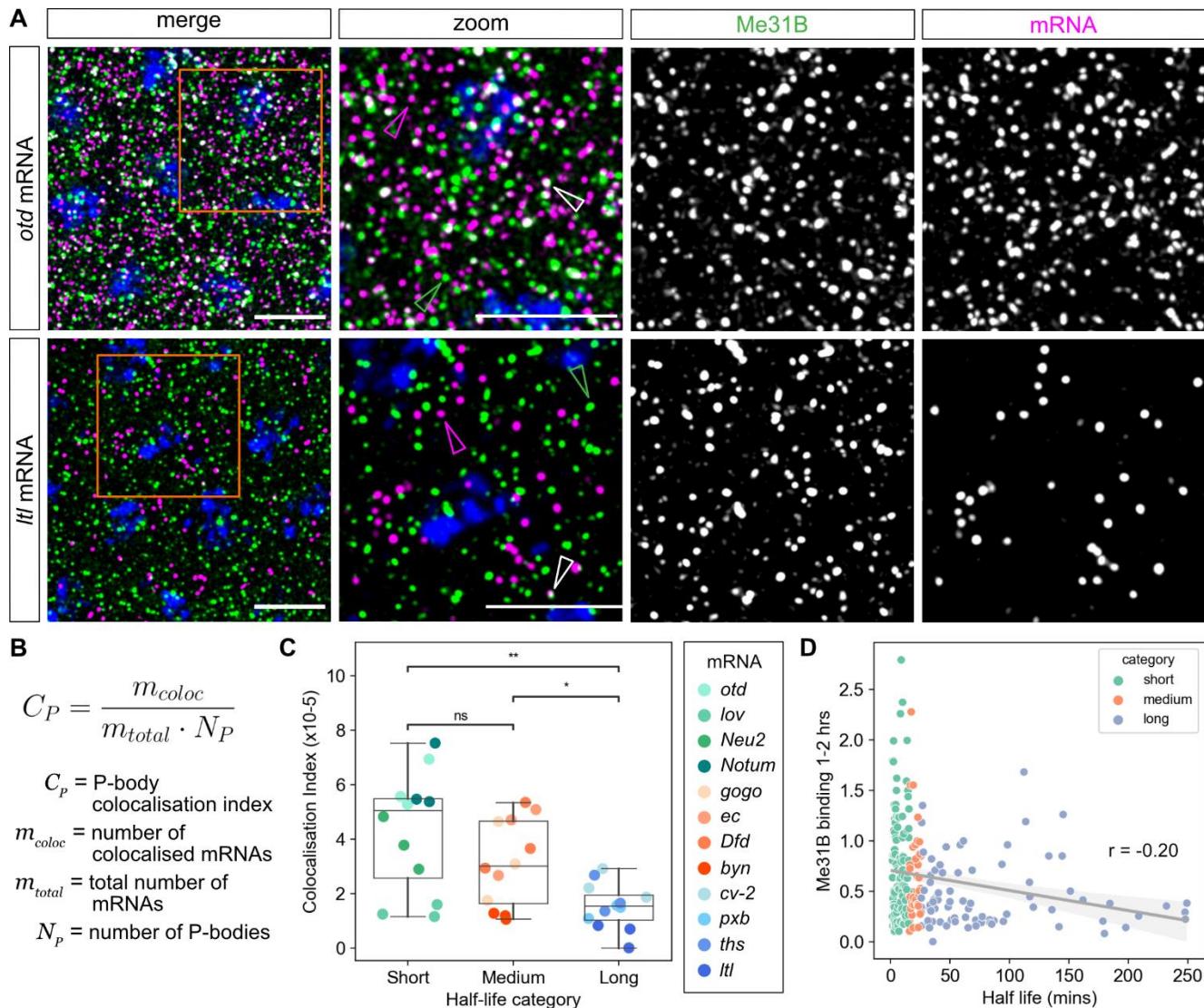
319 We find no correlation between compaction and mRNA length, as *brachyenteron* (*byn*) and  
320 *echinus* (*ec*) are the shortest and longest mRNAs tested, respectively. Taken together these results  
321 suggest that within early *Drosophila* development, the decay of zygotically expressed genes is not  
322 strongly correlated with translation efficiency or codon optimality, but unstable mRNAs tend to be slightly  
323 more compact than medium and long half-life transcripts.

324

### 325 **Embryonic P-bodies are associated with unstable mRNAs and enriched in 3' decay fragments**

326 Cytoplasmic P-bodies have been implicated in mRNA degradation and storage in *Drosophila* [52].  
327 Therefore, we investigated whether mRNAs with distinct stabilities are differentially localised to P-  
328 bodies. We visualised P-bodies using Me31B, a marker of P-bodies, including in *Drosophila* [53]. To  
329 detect Me31B we used a fly stock carrying a GFP-Me31B exon trap with GFP inserted into the Me31B  
330 locus [54]. We quantified both single mRNAs using smFISH and P-bodies labelled by GFP-Me31B in  
331 fixed embryos. The same set of eleven mRNAs described above was used in these experiments with

332 the addition of *Neu2*, a 1126 nt mRNA which was unsuitable for compaction analysis due to its short  
 333 length. Many GFP-Me31B foci were detected in the cytoplasm of early nc14 embryos (Figure 5A, Figure  
 334 S10A). These foci have a mean radius of 200 nm (Figure S10B), consistent with a previous observation  
 335 that P-bodies in the embryo are smaller than those in the oocyte [55].



336

337 **Figure 5: Short half-life mRNAs are more colocalised with P-bodies in the early embryo.**

338 (A) Confocal images of fixed, early nc14 embryos stained with smFISH probes for the indicated mRNAs  
 339 (magenta) and labelled GFP-Me31B P-bodies (green). Scale bars: 5  $\mu$ m. Images are maximum  
 340 projections of 7 slices, with higher magnification images of the highlighted regions (orange box) shown.  
 341 Individual mRNAs (magenta arrowheads), P-bodies (green arrowheads) and colocalised mRNA and P-  
 342 body signals (white arrowheads) are highlighted. (B) The P-body colocalisation index used to calculate  
 343 the normalised proportion of colocalised mRNAs, facilitating comparison between different mRNAs. (C)  
 344 Graph of the P-body colocalisation index for indicated mRNAs in early nc14. mRNAs are grouped by  
 345 half-life category, within which they are coloured by half-life as per Figure 4F with points representing  
 346 individual embryos. Both short and medium half-life mRNAs are significantly more colocalised with P-

347 bodies than long half-life mRNAs ( $p = 1.257 \times 10^{-3}$  and  $p = 1.640 \times 10^{-2}$ , respectively). Short half-life  
348 mRNAs are not significantly more enriched in P-bodies than medium half-life mRNAs ( $p = 4.168 \times 10^{-1}$ ).  
349 (D) Half-life versus Me31B binding data from 1-2 hour embryos [52] (Pearson's  $r = -0.20$ ,  $p = 1.5 \times 10^{-3}$ )  
350

351 For each mRNA tested, a proportion of the individual mRNA signals colocalise with P-bodies  
352 (Figure 5A, Figure S10A). As seen in Figure 5A, *orthodenticle* (*otd*) (also called *ocelliless*) mRNAs  
353 appear more highly colocalised with P-bodies than *Itl* mRNAs. As *otd* has a much shorter half-life than  
354 *Itl* (3 mins and 249 mins respectively), we examined whether this was a trend across the set of test  
355 mRNAs. To quantitate colocalisation, we used a colocalisation index that controls for variation in mRNA  
356 and P-body numbers between embryos (Figure 5B). This analysis reveals that the both the short and  
357 medium half-life mRNAs tested are significantly more colocalised with P-bodies than the long half-life  
358 mRNAs tested (Figure 5C). While the mean colocalisation index value for short half-life mRNAs is higher  
359 than that of the medium half-life mRNAs tested, this difference is not significant, due to higher variance  
360 in the colocalisation index of short half-life mRNAs as *lov*, a short half-life mRNA, has a particularly low  
361 colocalisation index.

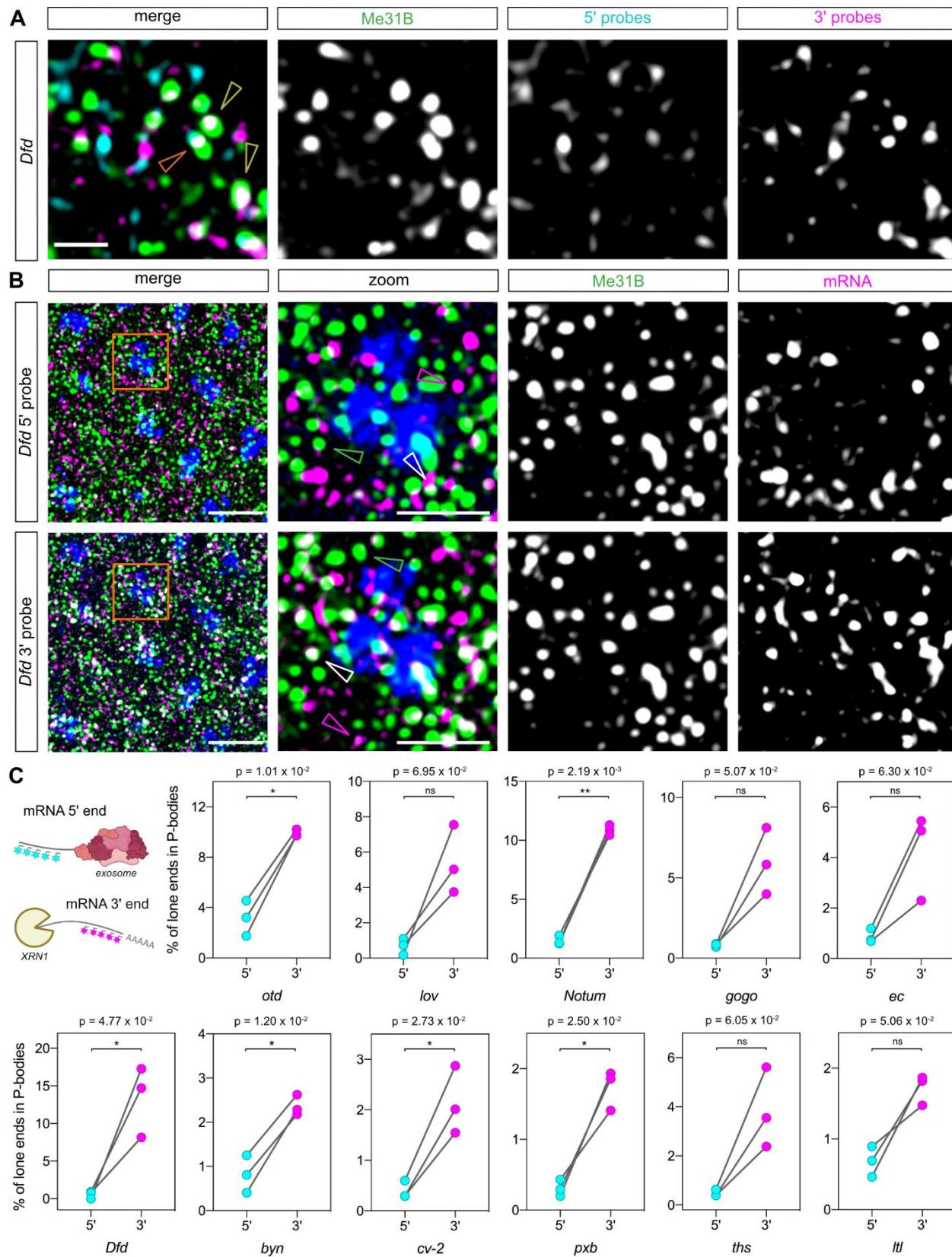
362 Given the difference in P-body colocalisation observed for some of the test mRNAs, we extended  
363 this analysis by using published Me31B RIP-seq data from the early *Drosophila* embryo [52]. This  
364 analysis reveals a relatively weak but significant negative correlation between Me31B interaction and  
365 mRNA half-life in 1-2 hr embryos (Figure 5D) and 2-3 hr embryos (Figure S10Ci). This negative  
366 correlation between the Me31B RIP-seq data and our model half-lives is no longer significant when the  
367 RIP-seq data from 3-4 hr embryos are used (Figure S10Cii), a later stage than we have imaged. These  
368 data are consistent with a previously reported negative correlation between Me31B binding and mRNA  
369 stability in the *Drosophila* embryo, when fold change in mRNA abundance was used as a proxy for  
370 mRNA stability [52]. Together, our imaging data and the negative correlation between RIP-seq  
371 interaction and mRNA half-life suggest that in the *Drosophila* embryo P-bodies may be sites of mRNA  
372 degradation for at least a subset of mRNAs.

373 In our dual-colour smFISH images we observed a proportion of unpaired 5' and 3' mRNA ends  
374 suggestive of degradation intermediates (Figure 4E). We detect more lone ends when we use 5' and 3'  
375 *otd* compaction probes, compared to alternating probes (Figure S9E), providing further support that  
376 some of the lone signals are due to mRNA degradation, as detection with alternating probes is more  
377 resistant to loss of mRNA 5' and 3' sequences. In addition, due to the short length of *Drosophila* mRNAs,  
378 we are using 24-30 probes in each detection set. Therefore, it is likely that loss of binding of only a small  
379 number of probes from the 5' or 3' set is enough to take the signal below the detection threshold,  
380 facilitating our detection of partly degraded mRNAs.

381 In order to determine if these 5' and 3' fragments co-localised with P-bodies we assessed  
382 whether the 5' and 3' probe sets colocalised with the GFP-Me31B P-body marker. An image of an early

383 nc14 embryo is shown for the *Dfd* mRNA in Figure 6A, revealing that some complete mRNAs (orange  
384 arrowhead) and lone 3' ends (yellow arrowhead) are colocalised with the P-body marker Me31B.  
385 However, colocalisation of lone 5' ends with Me31B is less evident. For clarity, an equivalent region of  
386 an early nc14 embryo is shown as 3 colour images with only either the 5' or 3' end of *Dfd* mRNAs,  
387 Me31B and DAPI (Figure 6B, Figure S11). For the analysis, we identified unpaired 5' and 3' ends as  
388 described above and assessed if there is an enrichment of either end in P-bodies (Figure S12A). In  
389 general, we do not see an excess of lone 3' ends compared to 5' ends across the mRNAs we tested  
390 (Figure S9). However, quantitation of the proportion of single 5' and 3' signals that localise to P-bodies  
391 reveals a general trend of more unpaired 3' ends in P-bodies, which is significant for over half the  
392 mRNAs investigated (Figure 6C). Similar results are obtained when the fluorophores on the *otd*, *Dfd*  
393 and *cv-2* 5' and 3' probes are reversed (Figure S12Bi). Furthermore, this trend is lost when we use  
394 alternating probes for *otd* (Figure S12Bii). Taken together these results suggest that the lone 3' signals  
395 detected in P-bodies are consistent with them being 5' to 3' mRNA decay intermediates. Additionally,  
396 comparison of the proportion of lone ends versus intact mRNAs in P-bodies (relative to the total number  
397 of each) reveals that in general the proportion of intact mRNAs and lone 3' ends in P-bodies is similar  
398 but there are more intact mRNAs than lone 5' ends (Figure S12C). This detection of intact mRNAs in P-  
399 bodies may support a storage role in addition to 5' to 3' decay (see Discussion). Together, these data  
400 suggest mRNA degradation can occur within P-bodies for at least some mRNAs in the early *Drosophila*  
401 embryo.

402  
403



404

405 **Figure 6: mRNA 3' end fragments are more associated with P-bodies than 5' ends.**

406 (A) Maximum projection (2 slices) of a confocal image showing smFISH staining of an early nc14 embryo  
407 with probes for *Dfd* 5' (cyan) and 3' (magenta) ends, GFP-Me31B (green) and DAPI staining (blue).  
408 Examples where both the 5' and 3' ends or only the 3' end is colocalised with P-bodies are indicated by  
409 orange and yellow arrowheads, respectively. Single channels for the smFISH and GFP-Me31B are  
410 shown with the merged image. Scale bar: 1  $\mu$ m. (B) As in (A) except the images (7 Z slices) show only  
411 one mRNA end (5' in the top panels, 3' in the lower panels) at a time for clarity. The mRNAs, GFP-  
412 Me31B and DAPI are shown in magenta, green and blue, respectively. A higher magnification image is  
413 shown as a merge and single channels, with individual mRNA ends (magenta arrowheads), P-bodies  
414 (green arrowheads) and colocalised mRNA end and P-body signals (white arrowheads) highlighted.  
415 Scale bars: 5  $\mu$ m in merge and 2  $\mu$ m in the higher magnification image. (C) Top left: Schematic showing  
416 how degradation by either pathway would result in a single fluorophore signal being detected.  
417 Quantification of the percentage of unpaired mRNA 5' and 3' ends with P-bodies relative to the total  
418 number of lone 5' or 3' ends.  $n = 3$  embryos, paired t-test used to determine significance with  $\alpha = 0.05$ .  
419 For absolute numbers of intact mRNAs and lone ends, see Figure S9A and S9B.  
420

## 421 Discussion

422 Here, using total RNA-seq time series data and Gaussian process regression, half-lives of  $\sim 260$  mRNAs  
423 in early *Drosophila* development were derived. Our data support widespread post-transcriptional  
424 regulation of gene expression in early development, as we show that shared transcription profiles give  
425 rise to a range of mature mRNA dynamics due to differences in degradation. The RNA-seq time series  
426 that we have generated is high resolution with additional time points and over an extended period of  
427 early embryogenesis compared to published data sets [24,56]. In addition, our libraries are total RNA-  
428 seq rather than poly(A) selected, facilitating detection of non-coding RNAs and unstable RNA species,  
429 such as co-transcriptionally spliced introns. Our RNA-seq data reveal how expression of different mRNA  
430 isoforms for a given gene varies across early embryogenesis and we highlight examples where isoform  
431 changes alter the protein sequence of specific domains, potentially impacting on function.

432 A major advantage of our approach is that it does not require transcription inhibition, which can  
433 affect mRNA stability, or mRNA labelling, which can be difficult to achieve *in vivo* and the labelling time  
434 can influence the estimates [19,20,57]. A different method that uses RNA-seq data to estimate mRNA  
435 half-lives has been described previously, which solves ODEs describing the RNA life cycle by adopting  
436 constraints on RNA kinetic rates [58]. An advantage of our approach is that, as Gaussian process  
437 regression is non-parametric, there is greater flexibility and sensitivity in the model to more accurately  
438 represent the variety of dynamics observed in the data. Additionally, it allows us to quantify uncertainty  
439 due to biological variation and measurement noise. Using Bayesian inference, we obtain posterior  
440 distributions for degradation parameters and thus quantify uncertainty about their possible values.

441 Disadvantages of our approach are firstly that it is not global as  $\sim 20\%$  of *Drosophila* genes  
442 expressed in our dataset do not contain introns. Secondly, even for mRNAs with introns, we only derived  
443 a proportion of transcript half-lives from the dataset due to strict filtering to ensure that there is signal in

444 both the intron and transcript expression time-series meaning genes with small introns and therefore  
445 poor signal would also be excluded. Thirdly, for genes with high degradation rates, there may be high  
446 uncertainty in the inferred degradation rate since the splicing rate and degradation rate estimates  
447 become difficult to disentangle (for simulations demonstrating the reach of the model see  
448 Supplementary Methods). Fourthly, the modelling requires some computational expertise in this area to  
449 implement on a new dataset. Potential solutions to overcoming these issues would be to generate a Pol  
450 II ChIP-seq time series for the transcription profiles that would allow intronless genes to be studied.  
451 Looser filtering could be applied to provide half-lives for more mRNAs, although this would potentially  
452 lower confidence in the estimates. Finally, the delay between the peak of the pre-mRNA and mature  
453 mRNA could be measured as a simpler approach for categorising stability, as we have shown that the  
454 stability of a transcript can be classified using this delay.

455 The half-lives we estimated for ~260 zygotic transcripts in the early embryo have a median of 16  
456 minutes. Previous half-life estimates of 7-14, 13 and 60 mins described for the zygotic *fushi tarazu*, *snail*  
457 and *hunchback* mRNAs, respectively, in the early *Drosophila* embryo [23,59,60] fall within the range of  
458 half-lives we observe. Moreover, the wide range of half-lives we estimate in the embryo suggests that  
459 mRNA stability is an important checkpoint in the regulation of gene expression. The median half-life we  
460 estimate is shorter than that of 73 minutes calculated for older (stage 12-15) *Drosophila* embryos, in a  
461 study that used a 4 hour pulse-chase labelling [37]. While the pulse-labelling timing may skew some of  
462 the half-life estimates [57], the shorter median half-life in the early embryo may reflect its rapid initial  
463 development. Early embryogenesis is characterised by short mitotic cycles [61] and fast rates of  
464 transcription [62] and translation [63], with the resulting localised gene expression patterns specifying  
465 three tissues along the dorsal-ventral axis in a time period of only 90 mins [64]. Therefore, mRNA  
466 degradation rates may be faster than at other stages to limit the perdurance of transcripts encoding  
467 factors affecting cell fate.

468 Gene ontology analysis revealed an enrichment among the short half-life mRNAs for those  
469 encoding transcription factors and cell adhesion proteins. This is consistent with transient localised  
470 expression of key transcription factors in the early embryo and the mRNAs encoding transcription factors  
471 commonly being unstable [17,37,38]. Future studies will be able to determine how particular mRNA half-  
472 lives contribute to patterning by exploiting the extensive characterisation of gene regulatory networks in  
473 the early *Drosophila* embryo [65].

474 Previous studies have shown that mRNAs exist in a more open conformation during translation,  
475 while untranslated mRNAs are more compact [44–46] regardless of whether they are stress granule  
476 associated [44,45]. We found a trend that the 5' and 3' ends are closer for shorter half-life mRNAs. A  
477 more compact structure may facilitate degradation as 5' to 3' decay involves communication between  
478 deadenylation and decapping factors [66]. Alternatively, the shorter distance between 5' and 3' ends

479 could reflect a transient interaction associated with degradation, which our smFISH snapshot images  
480 capture more frequently for the less stable mRNAs.

481 Codon identity and translation efficiency have previously been shown to be an important  
482 determinant of mRNA stability in bacteria, yeast, *Drosophila*, zebrafish and mammalian cells [41].  
483 Optimal codons, which are determined by codon bias in abundant mRNAs and the gene copy number  
484 of their cognate tRNA, lead to efficient translation and are enriched in stable transcripts [41]. However,  
485 our data suggest that codon optimality and translation efficiency are not major determinants of mRNA  
486 stability for early zygotic transcripts. A correlation between codon optimality and mRNA stability was  
487 observed for maternal mRNAs during the maternal to zygotic transition in the early *Drosophila* embryo,  
488 which likely contributes to clearance of maternal transcripts [67]. Optimal codons are also associated  
489 with stable mRNAs in late-stage *Drosophila* embryos, but not in neural tissues, potentially because  
490 mRNA stability regulation by RNA binding proteins dominates in the nervous system [43]. The effect of  
491 codon optimality may also be masked for early spatially regulated zygotic transcripts. This could be due  
492 to additional regulation by RNA binding proteins and miRNAs [5], a dependence on a particular  
493 distribution of non-optimal codons for instability [68] and/or tRNA abundance being a poor proxy for  
494 aminoacylated tRNA levels for a subset of tRNAs. In support of the latter, low aminoacylation of  
495 particular tRNAs has been observed in the mouse liver that may contribute to inefficient translation [69].

496 Our imaging data on the test set of mRNAs show that those with short half-lives tend to be more  
497 colocalised with the P-body marker Me31B than more stable mRNAs in the early embryo. Consistent  
498 with this, using published Me31B RIP-seq data from the early *Drosophila* embryo, we find a significant  
499 correlation between Me31B interaction and mRNA half-life across the set of ~260 mRNAs for which we  
500 estimated half-lives. The stronger association of short half-life mRNAs with P-bodies and our ability to  
501 detect mRNAs lacking their 5' end in P-bodies suggests that 5' to 3' mRNA decay can occur in P-bodies  
502 in the early *Drosophila* embryo. However, the majority of the lone 3' ends we detect are in the cytosol,  
503 suggesting that mRNAs can also undergo 5' to 3' decay outside of P-bodies. We also note that the  
504 localisation of mRNAs with P-bodies is variable as, within the short and medium half-life categories, the  
505 *lov* and *byn* mRNAs are less colocalised with P-bodies. This suggests that for these mRNAs in particular,  
506 degradation in P-bodies may only have a minor contribution to their turnover.

507 Although we generally detect similar proportions of 5' and 3' end fragments of a particular mRNA  
508 in the cytoplasm, there is weaker colocalisation of 5' end fragments with P-bodies. This observation  
509 suggests that 3' to 5' mRNA degradation by the exosome does not occur in P-bodies, consistent with  
510 components of the exosome being largely absent [4]. We also detect a similar proportion of intact  
511 mRNAs in P-bodies (relative to the total number in the cytoplasm), as we find for lone 3' ends. The  
512 presence of intact mRNAs in P-bodies may reflect an mRNA storage role. Therefore, we speculate that  
513 in the *Drosophila* embryo mRNAs enter P-bodies where they can undergo either: 1) 5' to 3' degradation

514 (hence the lone 3' ends detected), or 2) transient storage before exit back into the cytoplasm for  
515 translation.

516 A role for P-bodies in 5' to 3' decay is consistent with early studies in yeast following the discovery  
517 of P-bodies [70] and with later work in *Drosophila* suggesting that Me31B is involved in mRNA  
518 degradation in the embryo following zygotic genome activation [52] and P-bodies are sites of mRNA  
519 degradation in intestinal stem cells [71]. In addition, the Xrn1 exonuclease localises to P-bodies in yeast,  
520 *Drosophila* and mammalian cells [72]. However, P-bodies have been implicated in mRNA storage and  
521 translational repression in mature *Drosophila* oocytes [55] and Me31B represses translation of maternal  
522 mRNAs in *Drosophila* embryos prior to zygotic genome activation [52]. Moreover, many lines of evidence  
523 from other systems argue against a role for P-bodies in mRNA degradation. These include an absence  
524 of detectable mRNA decay intermediates either following purification of P-bodies [73] or based on a live  
525 imaging approach [47], mRNA degradation when P-body formation is disrupted [73] and the ability of P-  
526 body mRNAs to re-enter translation [74,75]. Although the sequencing data following P-body purification  
527 from human tissue culture cells provided evidence for mRNA storage, and do not support a role for P-  
528 bodies in bulk mRNA degradation [73], we note that two pieces of data are potentially consistent with  
529 some degradation occurring in P-bodies. Firstly, there is a weak correlation between mRNA P-body  
530 enrichment and half-life, and secondly a 3-fold difference in the median half-lives of the most strongly  
531 enriched versus depleted P-body mRNAs was observed [73].

532 We speculate that P-bodies are involved in both storage and degradation in an mRNA dependent  
533 manner, with features of an individual mRNA as well as the proteins present in P-bodies at a particular  
534 developmental time influencing which function dominates. In support of this, it is known that there are  
535 changes in P-bodies during *Drosophila* development, for example from being large and viscous in the  
536 oocyte to smaller, more dynamic structures in the early embryo [55]. Moreover, at the maternal-to-  
537 zygotic transition some P-body proteins are degraded, including the Cup translational repressor protein,  
538 which may increase the prevalence of mRNA decay in P-bodies [52]. Our data suggest that the  
539 degradation of mRNAs in P-bodies contributes to the post-transcriptional regulation of zygotic mRNAs  
540 in the embryo. Future studies exploiting the method developed for determining the protein and RNA  
541 contents of purified P-bodies [73], along with the power of *Drosophila* genetics and single molecule  
542 imaging, will reveal how P-bodies impact on mRNA stability or storage and cell fate decisions during  
543 development.

544

545

## 546 **Materials and Methods**

547

## 548 **Biological methods**

549

550 **Fly stocks**

551 All stocks were grown at 25°C and maintained at 20°C for experiments on standard fly food media (yeast  
552 50g/L, glucose 78g/L, maize 72g/L, agar 8g/L, 10% nipagen in EtOH 27mL/L and propionic acid 3mL/L).  
553 The following fly lines were used in this study,  $y^1 w^*$  (BDSC Stock #6599),  $y^1 w^*$ ; P{His2Av-mRFP1}II.2  
554 (BDSC Stock #23651) and  $y^1 w^*$ ; P{w[+mC]=PTT-GB}me31B[CB05282] (BDSC Stock #51530).

555

556 **Staging and collection of embryos for RNA-seq**

557 Flies carrying His-RFP were allowed to lay on apple juice agar plates in small cages for 1 hour. Embryos  
558 were dechorinated in 50% bleach (2.5% final concentration of sodium hypochlorite diluted in distilled  
559 water) for 3 minutes and washed thoroughly in distilled water. Individual embryos were carefully  
560 transferred into a droplet of halocarbon oil (Sigma-Aldrich; a mix of 700 and 27 oil at a ratio of 1:4) on a  
561 rectangular coverslip (Deltalab, 24X50mm, Nr. 1) and inverted over a cavity slide (Karl Hecht). Embryos  
562 were visualised and imaged with a Leica optigrid microscope at 20X magnification using a Texas red  
563 filter. Embryos were timed following the observation of a nuclear division, an image was taken and the  
564 embryo was immediately picked out of the oil droplet with a pipette tip and transferred to Eppendorf  
565 tubes containing 50 $\mu$ L TRIzol Reagent (Invitrogen). Single embryos were crushed and homogenised  
566 using a pipette tip and an additional 450 $\mu$ L Trizol added. Samples were immediately snap frozen in  
567 liquid nitrogen and stored at -80°C until processing for nucleic acid extraction.

568 Ten timepoints were collected spanning early *Drosophila* embryonic development from nc11  
569 through to cephalic furrow formation (Table 1). Embryos were collected 5 minutes after nuclear division  
570 for nc11 and nc12, 5 and 15 minutes following the nc13 nuclear division and every 15 minutes following  
571 the nc14 nuclear division as well as embryos that showed clear cephalic furrow formation. This yielded  
572 samples covering every 10-15 minutes through development from nc11 to cephalic furrow formation.  
573 The internuclear distance of 15-20 nuclei pairs per embryo was measured in Fiji and normalised to the  
574 whole embryo length to obtain an average internuclear distance per embryo (Figure S1A and B). This  
575 was compared to the internuclear distance of embryos of known stages to accurately confirm the nuclear  
576 cleavage stage and age of embryos. All embryos were collected at 20°C with approximate time after  
577 egg lay in minutes shown in Table 1.

578

579 **Table 1: *Drosophila* embryo samples collected for RNA-seq time course**

Sample collection time information	Sample stage ID	Approximate time after egg lay at 20°C (mins)
------------------------------------	-----------------	---

nc11 + 5mins	nc11	95
nc12 + 5 mins	nc12	105
nc13 + 5 mins	nc13A	115
nc13 + 15 mins	nc13B	125
nc14 + 15 mins	nc14A	145
nc14 + 30mins	nc14B	160
nc14 + 45mins	nc14C	175
nc14 + 60 mins	nc14D	190
nc14 + 75 mins	nc14E	205
CF	CF	>220

580

581 **Nucleic acid extraction and embryo genotyping**

582 Samples stored in Trizol (Invitrogen) were used for RNA and DNA extraction performed according to  
583 the manufacturer's protocol and resuspended in 10µL (RNA) or 20µL (DNA) nuclease free water.  
584 Extracted DNA was PCR amplified to sex the embryos by using Y chromosome specific primers to a  
585 region of the male fertility factor gene kl5, forward primer 5' GCTGCCGAGCGACAGAAAATAATGACT  
586 3' and reverse primer 5' CAACGATCTGTGAGTGGCGTGATTACA 3' [24] and control primers to a  
587 region on chromosome 2R forward primer 5' TCCCAATCCAATCCAACCCA 3' and reverse primer 5'  
588 CCTACCCACAAGCAACCAACC 3'. PCR reactions were performed in triplicate.

589 Total RNA was treated with TURBO DNA-free Kit Dnase (Invitrogen) and depleted of rRNA using  
590 the Ribo-Zero Magnetic Kit HMN/Mouse/Rat 24 Rxn (Illumina; Cat# MRZH11124) according to the  
591 manufacturer's protocol using a low input protocol with 2-4µL rRNA removal solution yielding a 20µL  
592 final sample volume added to 90µL magnetic beads. Beads were resuspended in 35µL resuspension  
593 solution and ribo-depleted total RNA was ethanol precipitated and resuspended in 18µL FPF mix prior  
594 to RNA-seq library preparation.

595

596 **RNA-seq library preparation and sequencing**

597 Three female embryos from each time point were used as replicates to make 30 individual RNA-seq  
598 libraries. Individual total RNA-seq libraries were prepared from ribo-depleted RNA using a TruSeq

599 stranded library prep kit (Illumina) according to the manufacturer's protocol. Unique dual index adaptors  
600 were used for each library and they were pooled in equimolar concentration and run across 8 lanes on  
601 the flow cell of the HiSeq 4000 to obtain paired end sequence reads. The average number of reads  
602 obtained per library was 105 million reads.

603

604 **Embryo fixation and smFISH**

605 Flies were allowed to lay on apple juice agar plates in small cages for 2 hours at 25°C. After ageing for  
606 another 2 hours, 2-4 hour old embryos were dechorinated in 50% bleach for 3 minutes and washed  
607 thoroughly in distilled water. Embryos were fixed as previously described [76] and stored in methanol at  
608 -20°C until required. Fixed embryos were placed in Wheaton vials (Sigma, Z188700-1PAK) for the  
609 smFISH reaction as described previously [77]. mRNA targets were detected in embryos using smiFISH  
610 probes designed to exonic sequence with 5' end X flap sequences [78] and using secondary detection  
611 probes labelled with Quasar 570 or Quasar 670 fluorophore dyes (LGC Biosearch Technologies). Probe  
612 sequences are listed in Supplementary Table 2. DAPI (500µg/ml) was added to the third of the final four  
613 washes of the protocol at a concentration of 1:1000 and embryos were mounted onto slides in Prolong  
614 Diamond to set overnight before imaging. To visualise the membrane to age the embryos a mouse  $\alpha$ -  
615 Spectrin antibody (DSHB, 3A9 (323 or M10-2)) with an Alexa Fluor 647 Donkey anti-Mouse IgG (H+L)  
616 Highly Cross-Adsorbed Secondary Antibody (Thermo Fisher Scientific, A-31571) was used or a  
617 brightfield image was taken.

618 For compaction experiments at least 24 probes were designed to each end of the mRNA (5' and 3')  
619 separated by at least 1.3kb. As a control, fluorophore dyes were switched and the images from stained  
620 embryos analysed and quantified. Additional controls for *otd* and *lov* used adjacent probes with  
621 alternating Quasar dyes to determine the precision of detection of single mRNAs.

622

623 **Confocal microscopy of fixed embryos**

624 A Leica TCS SP8 gSTED confocal was used to acquire images of the transcription sites (TSs), single  
625 mRNAs and P-bodies within cells of fixed embryos using a 100x/ 1.3 HC PI Apo Cs2 objective with 3X  
626 line accumulation and 3X zoom for compaction and P-body colocalisation experiments, and 2X zoom  
627 for quantifying mRNAs for the half-life validation. Confocal settings were ~0.6 airy unit pinhole, 400 Hz  
628 scan speed with bidirectional line scanning and a format of 2048 x 2048 or 4096 x 4096 pixels. Laser  
629 detection settings were collected as follows: PMT DAPI excitation at 405nm (collection: 417-474nm);  
630 Hybrid Detectors: AlexaFluor 488 excitation at 490nm (collection: 498-548nm), Quasar 570 excitation  
631 at 548nm (collection: 558-640nm) and Quasar 670 excitation at 647nm (657-779nm) with 1-6ns gating.

632 All images were collected sequentially and optical stacks were acquired at system optimised  
633 spacing. Imaging of the membrane using brightfield or anti-Spectrin antibody at the mid-sagittal plane

634 of the embryo with 40x objective at 0.75X zoom and 1024 X 1024 format was used to measure the  
635 average length of membrane invagination from at least 5 cells. These measurements were used to  
636 select embryos of a similar age in early nuclear cycle 14 (10  $\mu$ m membrane invagination). For all  
637 analysis, 3 separate embryos were imaged and quantified as independent replicates. smFISH images  
638 were deconvolved using Huygens professional deconvolution software by SVI (Scientific Volume  
639 Imaging). By deconvolving images taken on a Leica SP8 confocal we estimate that our lower detection  
640 limit is ~120nm.

641

## 642 **Image analysis**

643 The spot detection algorithm Airlocalize [79] was used to detect and quantify TSs, single mRNAs and  
644 P-bodies within confocal microscopy images. This software fits a 3D gaussian mask and gives the  
645 coordinates in X, Y and Z of each spot and its intensity. Z stack images were first subsetted to detect  
646 TSs within the range of Z slices around the nuclei. Images were then processed again to detect single  
647 mRNAs in the full image. The TS data was then used to remove these high intensity spots from the  
648 single mRNA data. Detection of 5' and 3' single mRNA ends and P-bodies was performed separately  
649 on each corresponding channel image as appropriate.

650

## 651 **Half-life validation**

652 For validation of half-lives as previously described [23], embryos were imaged at various time points  
653 during the 13th nuclear division (Figure S4) using the DAPI channel and reference movies of His-RFP  
654 [77] to carefully time the images. Single mRNAs were quantified using Airlocalize and the number per  
655 cell was calculated by dividing by the total number of pre-division cells in the images. The counts per  
656 cell were fitted with an exponential function, from which the half-life was determined. The signal to noise  
657 ratio in the data was then calculated from the change in the mean over the time course, divided by the  
658 average variance in mRNA numbers at each timepoint with sufficient data.

659

## 660 **Computational methods**

661

## 662 **RNA-seq data processing and data filtering**

663 The RNA-seq data were processed at the transcript level by alignment-free methods using Kallisto [80]  
664 and the *Drosophila melanogaster* Ensembl v96 reference transcriptome to infer transcript expression  
665 levels for modelling mature mRNA dynamics. Transcripts were filtered to remove any that had <1 TPM  
666 (transcripts per million) across the time course yielding 18159 remaining transcripts. The transcript-level  
667 reads were then filtered for dynamic transcripts using GPcounts [81]. This approach fits the data for  
668 each gene using a GP with a dynamic kernel, and a GP with white noise kernel which exhibits no

669 variation over time. The transcripts where the dynamic kernel is a better fit, measured via likelihood ratio  
670 test, are then extracted. For the transcript data this reduced the numbers of mRNAs from 18159 to 8791.

671 The whole-embryo total RNA-seq dataset was also processed at the gene level in order to  
672 quantify the intronic reads, by aligning data to BDGP6 (dm6) using STAR with default parameters.  
673 FeatureCounts was used to get the counts data for exons and introns, respectively. Modified RPKM  
674 (reads per kilobase of transcript per million reads mapped) normalisation was applied to exon and intron  
675 counts data, where the total mapped reads for each library were used to address the sequencing depth  
676 for exon and intron counts from the same sample yielding 11,587 genes with a detectable level of  
677 expression (RPKM > 0).

678 To model the pre-mRNA dynamics, any genes without introns, or with zero intronic reads across  
679 all timepoints were removed to give a set of 5035 genes and the intron sizes were then used to obtain  
680 length-normalised reads. The intronic read counts are divided by the total intron length for each gene  
681 and the exonic expression level is similarly normalised. For modelling the mature mRNA dynamics the  
682 transcript-level alignment was used. A set of strictly zygotic transcripts were extracted from the dynamic  
683 dataset (n = 8791) by filtering for transcripts with TPM < 0.5 at the first time point (t = 95) to give a set  
684 of 593 zygotic transcripts which were used in subsequent analysis. For the GP model, transcripts were  
685 subjected to a further filtering step where the correlation between pre-mRNA and mRNA was computed  
686 to extract transcripts where the correlation was above 0.4. For more details on filtering see  
687 Supplementary Methods.

688

#### 689 **Analysis of intronic read coverage**

690 BAM files were used to calculate the read coverage for introns and exons to the  
691 *Drosophila\_melanogaster.BDGP6.32.107.gtf* annotation using the superintronic package in R with  
692 default parameters [82].

693

#### 694 **Validation of intronic data with NET-seq data**

695 In order to validate that the intronic data is representative of nascent zygotic gene transcription, NET-  
696 seq data from early embryos was used [29]. Both the NET-seq and intronic data were length normalised  
697 and a Spearman's Rank correlation analysis was used to analyse the relationship.

698

#### 699 **Modelling**

700 We model dependence between pre-mRNA,  $p(t)$ , and mature mRNA,  $m(t)$ , through a Gaussian  
701 processes regression which follows dynamics of an ODE of the form

702

$$\frac{dm(t)}{dt} = Sp(t) - Dm(t),$$

703 where  $p(t)$ , is assumed to be a Gaussian process with RBF kernel [31,32]. This differential equation  
704 can be solved in closed form and it can be shown that  $m(t)$  is also a Gaussian process with a certain  
705 kernel. For more details and specification of this kernel we refer to Supplementary Methods. As the  
706 results,  $m(t)$  and  $p(t)$  can be modelled jointly as a Gaussian process regression with a block kernel  
707 which depends on biologically interpretable parameters such as  $S$  and  $D$ . It is assumed that we observe  
708  $m(t)$  and  $p(t)$  at discrete times with measurement noise terms which have variances  $\sigma_m^2$  and  $\sigma_p^2$  for  
709 mRNA and pre-mRNA respectively. Thus, we have six parameters which we estimate: two parameters  
710 of RBF kernel ( $l$  – lengthscale,  $\sigma^2$  – variance, which correspondingly define smoothness and amplitude  
711 of possible functions underlying pre-mRNA dynamics), two parameters  $S$  and  $D$ , which describe the  
712 relationship between mRNA and pre-mRNA, and two measurement noise variances  $\sigma_m^2$  and  $\sigma_p^2$ .

713 We assign priors to these six parameters and use the L-BFGS-B algorithm to find maximum a  
714 posteriori (MAP) point estimates. Further, we perform sampling from the posterior distribution using  
715 Metropolis-adjusted Langevin algorithm (MALA). By using gradient information this algorithm allows  
716 exploring posterior distribution more efficiently than standard MCMC methods. After inferring the  
717 posterior distribution using sampling with MALA, we can quantify uncertainty about all six parameters of  
718 the model and uncertainty about the underlying functions for pre-mRNA and mRNA dynamics. In  
719 Supplementary methods Figure S1 we demonstrate the performance of the model and inference  
720 methods on simulated data: degradation rates ranging between 0.003 and 0.05 (corresponding to half-  
721 lives of 231.0min and 13.8min, respectively) are estimated accurately by both MAP and MALA  
722 approaches. The details of the implementation, including the assumptions on prior distributions, are  
723 discussed in Supplementary Methods.

724 From parameter estimates of  $D$ , half-lives were obtained using the following relationship:  
725

$$726 \quad t_{1/2} = \frac{\ln(2)}{D}$$

727

728 Transcripts were then grouped into short, medium and long half-life groups, setting the  
729 boundaries at 15 and 25 minutes. Any transcripts with a half-life longer than 250 minutes were removed  
730 from the dataset.

731 Python implementation of the model is available from  
732 [https://github.com/ManchesterBioinference/GP\\_Transcription\\_Dynamics](https://github.com/ManchesterBioinference/GP_Transcription_Dynamics).

733

734 **Clustering**

735 The intronic data ( $n = 5035$ ) was z-transformed and clustered using GPclust, a package specifically  
736 designed for clustering noisy time series data using Gaussian processes [39]. Intronic clusters of interest  
737 exhibiting a range of expression profiles were selected (clusters 2 and 5) (Figure S5). The zygotic  
738 transcripts ( $n = 593$ ) corresponding to the genes in each selected intronic cluster were then normalised  
739 and clustered (Figure 3; Figure S6). Summary statistics for the half-lives of the genes in each zygotic  
740 cluster were then computed for clusters with  $>2$  transcripts with estimated half-lives. A list of the  
741 transcripts for intronic clusters 2 and 5 and their corresponding zygotic clusters can be found in  
742 Supplementary Table 3.

743

744 **Analysis of time delays**

745 Delays between peak of pre-mRNA and mature mRNA time series from the zygotic set ( $n = 593$ ) were  
746 estimated by fitting a Gaussian process with RBF kernel to each time series. 100 samples from each  
747 GP were taken and the delay between the peak of each sampled function of premature and mature  
748 mRNA were computed to provide estimates of the delay with uncertainty. Any transcripts with delays  $\leq$   
749 0, or with mature mRNA profiles peaking at the final timepoint ( $t = 220$ ), were removed. Transcripts were  
750 then grouped into short, medium and long delay groups, setting the boundaries from the 33% and 66%  
751 quantiles of the data (17.55 and 36.16 mins respectively). A gene was classified as short if there was  
752 90% probability that the delay for that gene was in the short delay interval. All statistical analysis was  
753 carried out in Python using the scipy, sklearn and statannot libraries.

754

755 **Gene ontology analysis**

756 Gene ontology analysis was conducted using Gorilla [83]. Enrichment of short and long half-life genes  
757 was performed using the half-life set as the target set and the entire group of dynamic genes from the  
758 RNA-seq dataset ( $n = 8791$ ) as the reference set with default parameters.

759

760 **Codon usage and translation efficiency analysis**

761 The codon stabilisation coefficient (CSC) value was calculated for each codon as previously described  
762 [43,84]. The CSC is equivalent to the Pearson correlation coefficient, calculated by plotting the  
763 frequency of each codon per transcript within our dataset against its half-life. Classification of optimal  
764 *Drosophila* codons used are as in [43]. A chi-square test of association between optimal and non-optimal  
765 codons in positive and negative CSC groups was determined. The codon optimality score was  
766 determined by adding the proportion of optimal codons within each transcript. Transcripts were grouped  
767 by their half-life category and an independent t-test was used to determine significance in codon

768 optimality between groups. Translation efficiency data was obtained from previously published data of  
769 2-3 hour embryos [42]. 3'UTR and transcript lengths were obtained from Flybase [85].  
770

### 771 Quantification of mRNA end-to-end distance

772 mRNA compaction, the distance between the 5' and 3' ends of the transcripts, was analysed using  
773 smFISH images where the 5' and 3' ends are bound by probes labelled with different fluorophores. After  
774 quantifying the number and position of the mRNA ends in both channels and removing transcription  
775 sites (see Image analysis), the spot position data was analysed with a custom Python script to find  
776 optimal spot pairs by solving a paired assignment problem. The distance between  $n$  5' spots and  $m$  3'  
777 spots are computed and stored as a distance matrix. The optimal assignment of 5' and 3' pairs is then  
778 found by minimising this distance matrix to give a set of paired spots with a minimum total distance  
779 between all pairs. Spot pairs are then filtered for distances less than 300 nm where the ends are  
780 considered to be colocalised and belonging to the same RNA. This 300 nm upper threshold was selected  
781 as described in a previous study [46]. For all colocalised 5' and 3' spots the distribution of distances was  
782 then analysed and summary statistics extracted.  
783

### 784 Analysis of mRNA colocalisation with Processing bodies

785 mRNA localisation within Processing bodies (P-bodies) was determined from confocal images using a  
786 custom script in Python. This script uses the position data for the mRNAs and P-bodies output from  
787 Airlocalize and calculates the distance between a given mRNA and every P-body. The minimum  
788 distance is then selected so that an mRNA is assigned to its closest P-body. If this distance is less than  
789 200 nm (a typical radius of a P-body) then the RNA is considered to be colocalised with the P-body. The  
790 proportion of mRNAs located within and outside of P-bodies is then analysed to determine whether a  
791 given gene is enriched within P-bodies in the cytoplasm. In order to do this, we derived the P-body  
792 colocalisation index, a measure of the degree of colocalisation with P-bodies of an mRNA of interest:  
793

$$794 C_P = \frac{m_{coloc}}{m_{total} \cdot N_P}$$

795  
796  
797 Where  $C_P$  is the P-body colocalisation index,  $m_{coloc}$  is the number of mRNAs colocalised with P-bodies,  
798  $m_{total}$  is the total number of mRNAs and  $N_P$  is the number of P-bodies.

799 For analysis of unpaired ends, any 5' or 3' spot which was unpaired from the optimal assignment,  
800 or was more than 300 nm away from its assigned pair, was considered. The colocalisation of these with  
801 P-bodies was then analysed using a more conservative threshold of 150 nm, to ensure a sufficient

802 proportion of the mRNA was located inside the P-body. The enrichment of unpaired ends in P-bodies  
803 was then derived by dividing the number of unpaired ends in P-bodies by the total number of unpaired  
804 ends for each channel.

805

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815

## 816 **Author Contributions**

817 Conceptualisation, L.F.B, J.C.L, Y.S, M.R and H.L.A; Investigation, L.F.B, J.C.L, Y.S; Software, Y.S.,  
818 A.A; Writing; L.F.B, J.C.L, H.L.A, Y.S; Funding Acquisition, M.R. and H.L.A.

819

## 820 **Declaration of interests**

821 The authors declare no competing interests.

822

## 823 **Availability of data and materials**

824 RNA-seq data are deposited in ArrayExpress under accession number: E-MTAB-11580. Python  
825 implementation of the model is available from:

826 [https://github.com/ManchesterBioinference/GP\\_Transcription\\_Dynamics](https://github.com/ManchesterBioinference/GP_Transcription_Dynamics).

827

828

829

830

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1061 **Supplementary Data**

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1063 Supplementary Figures S1-S12

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1065 Supplementary Table 1: Table of half-lives

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1067 Supplementary Table 2: Table of smFISH probes used in this study

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1069 Supplementary Table 3: Intronic and zygotic clusters and IDs

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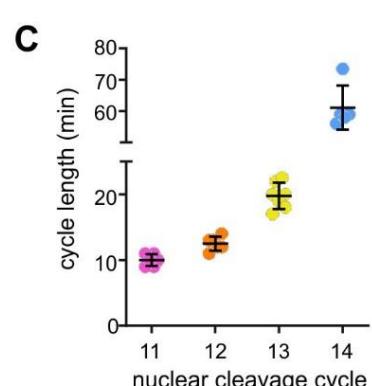
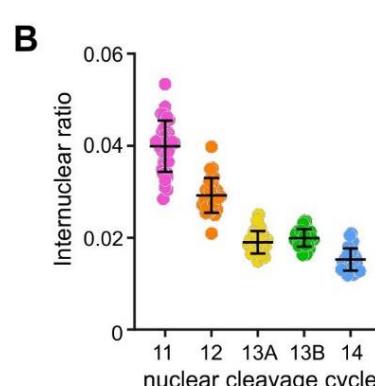
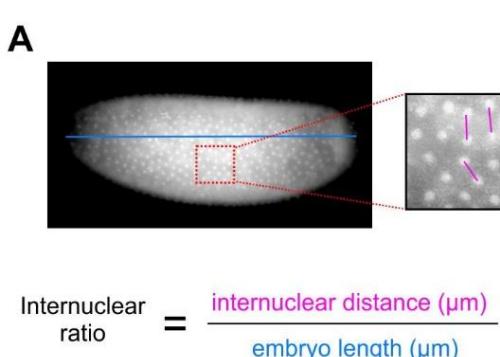
1071 Supplementary Methods: Filtering of the genes, model formulation and Bayesian inference with MCMC,  
1072 simulation studies

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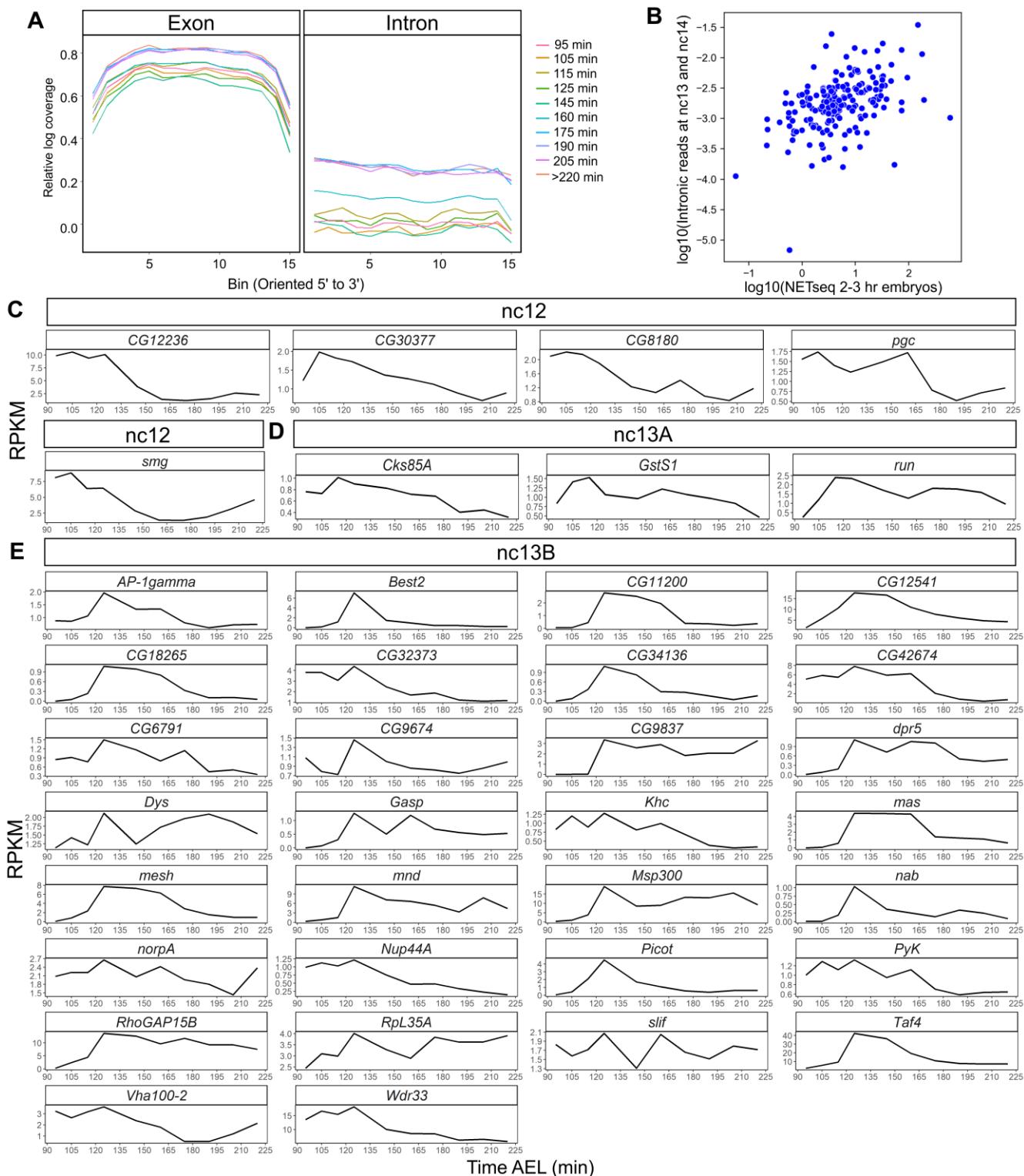
1076 **Supplementary Figures**



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1078 **Figure S1. Staging of early embryos for RNA-seq.** (A) Images of embryos were captured immediately  
1079 prior to collection and the internuclear distance and embryo length were measured for each to give an  
1080 internuclear ratio. (B) The internuclear ratio at nuclear cleavage cycles (nc) can be used to accurately  
1081 stage embryos. (C) Timing of each nc at 20°C used in experiments.

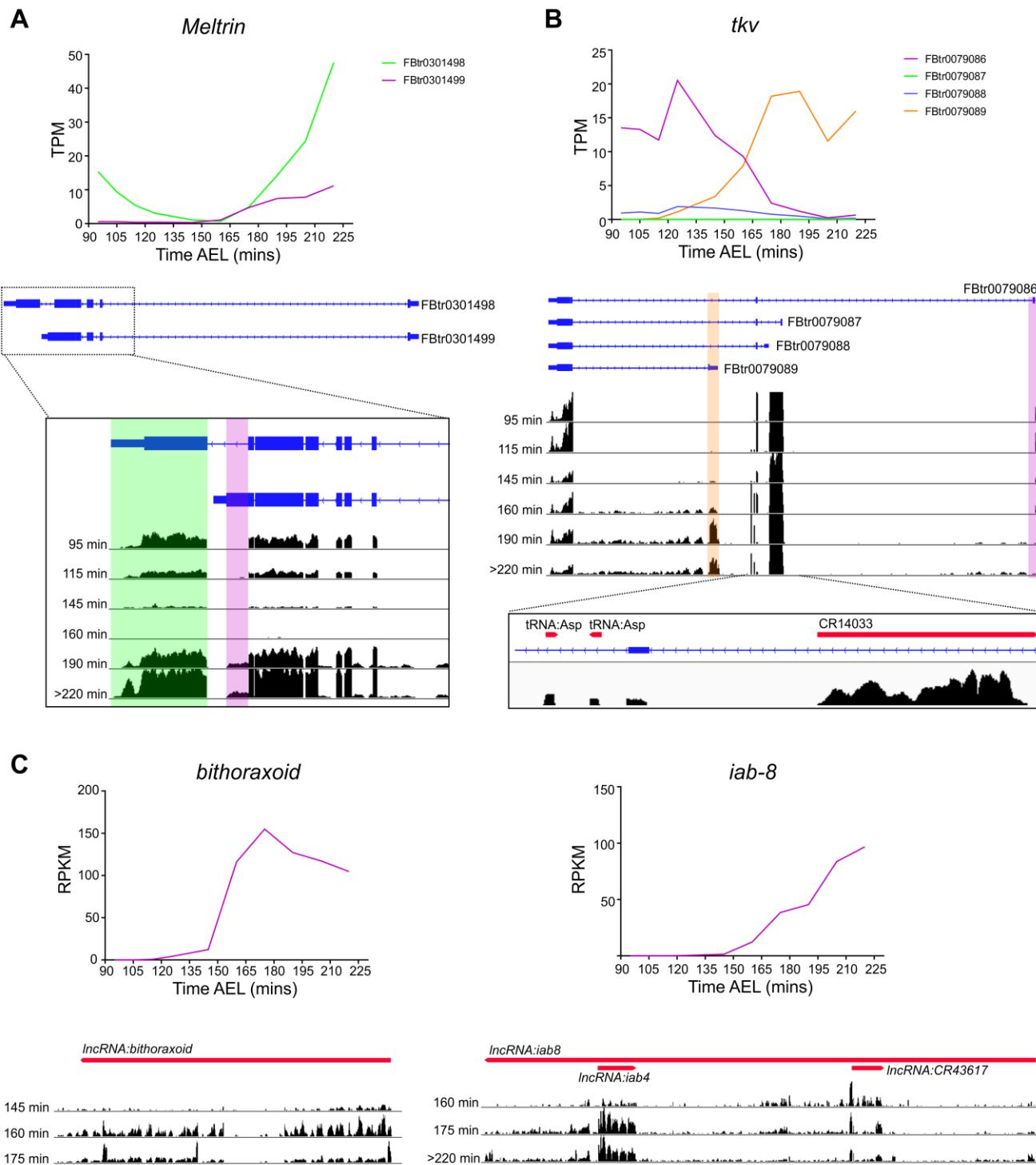
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1084 **Figure S2. Read coverage and pre-mRNA profiles of early zygotic genes.** (A) Binned read coverage  
 1085 across exons and introns for one replicate for each of the time points within the time series. (B)  
 1086 Scatterplot of our RNAseq reads versus NET-seq read counts showing a relationship (Spearman's Rank  
 1087 correlation  $\rho = 0.46$ ,  $p = 5.1 \times 10^{-11}$ ). Intronic read counts (RPKM) of genes that show early transcription

1088 in our dataset, grouped depending on peak expression at (C) nc12 (105 min), (D) nc13A (115 min) or  
1089 (E) nc13B (125 min), based on timings at 20°C.



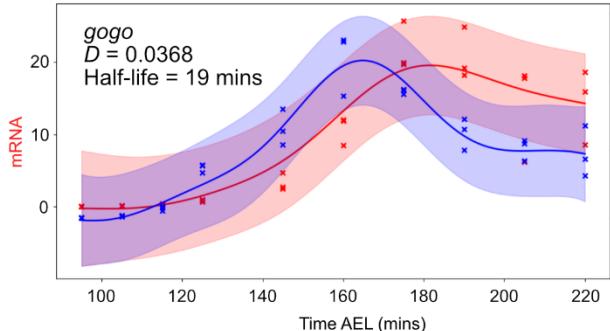
1091 **Figure S3. Examples of alternative isoform and non coding RNA expression during development**  
1092 **in *Drosophila* embryos.** (A) Quantification of the two different transcripts (TPM) of the *Meltrin* gene.  
1093 The FBtr0301499 isoform (purple) is not detected in embryos <160 min AEL but is detected at later time  
1094 points in addition to the FBtr0301498 (green) transcript. Mapped reads are shown below with a region  
1095 highlighted in purple depicting the increase in the alternative transcript at time point 190 and 220 min.  
1096 (B) As in (A) but for the gene *tkv*. Transcription of the FBtr0079086 (purple) and FBtr0079089 (orange)

1097 isoforms switches during the time course of development, as highlighted on the mapped reads below.  
1098 Expression of non coding RNAs, including two copies of the tRNA-Asp and a pseudogene (CR14033),  
1099 overlap the *tkv* locus as shown in the expanded region below from time point 220 min. (C) Expression  
1100 of the non-coding RNAs *bithoraxoid* and *iab-8*. Gene level counts (RPKM) show dynamic expression  
1101 across the time course of these two non-coding genes. Gene regions for the ncRNAs (red) are shown  
1102 with the genome browser tracks below.  
1103

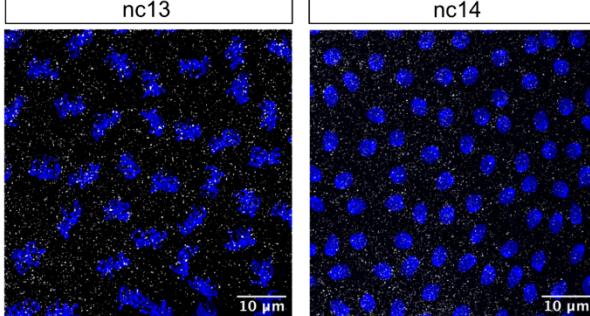
**A**

Gene	Transcript ID	$D$	MAP half-life (mins)	Lower credible interval (mins)	Upper credible interval (mins)
<i>lov</i>	FBtr0072458	0.0752	9	6	15
<i>cv-2</i>	FBtr0071610	0.0246	28	17	937

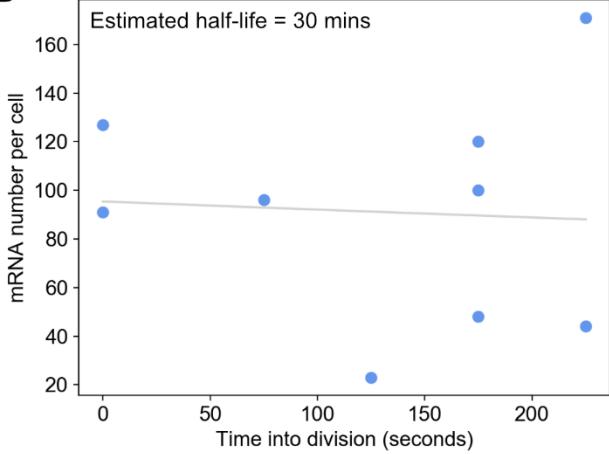
**B**



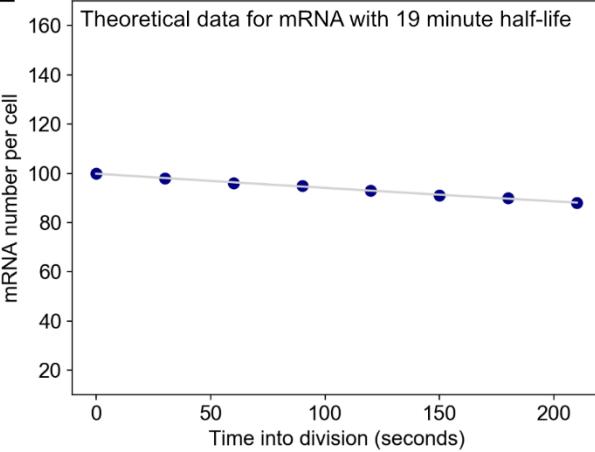
**C**



**D**



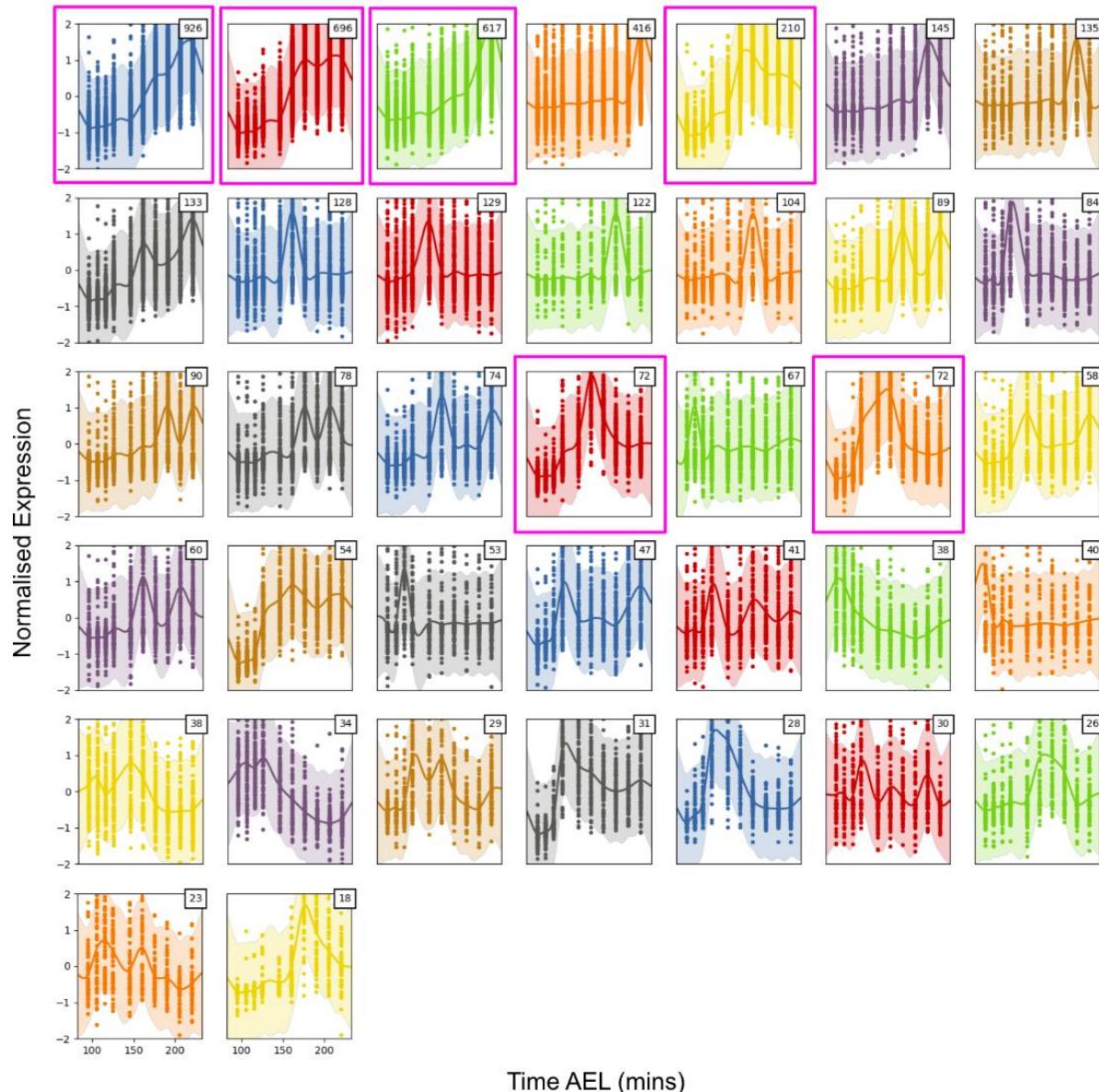
**E**

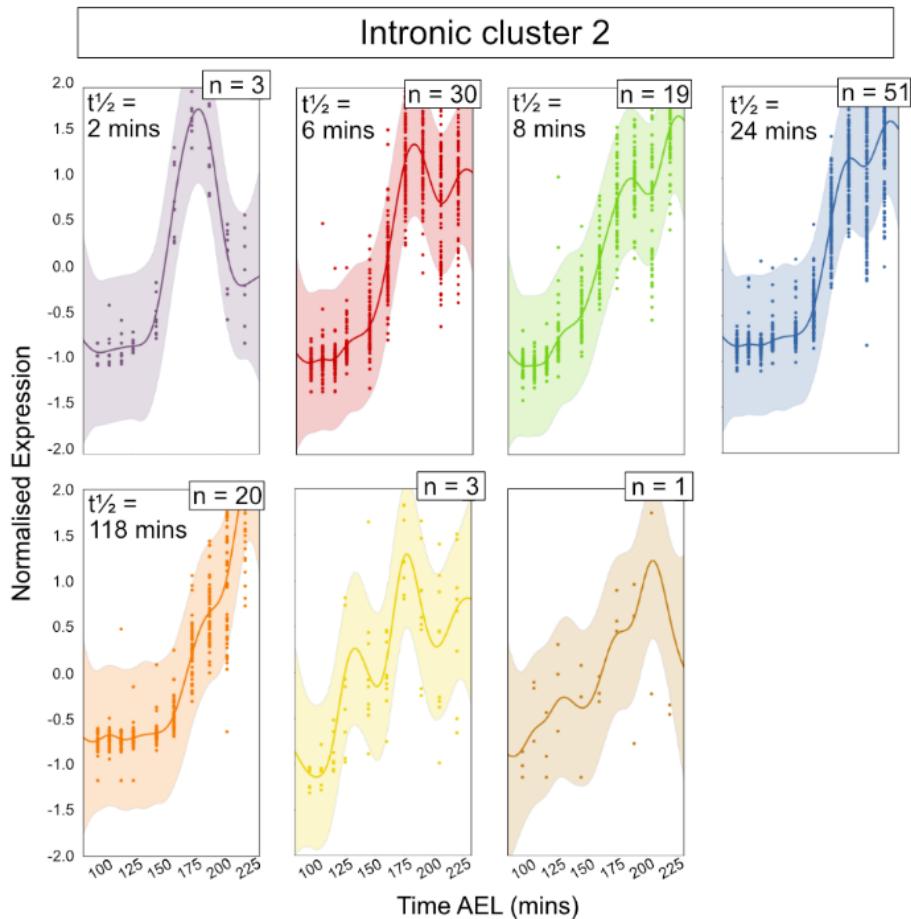


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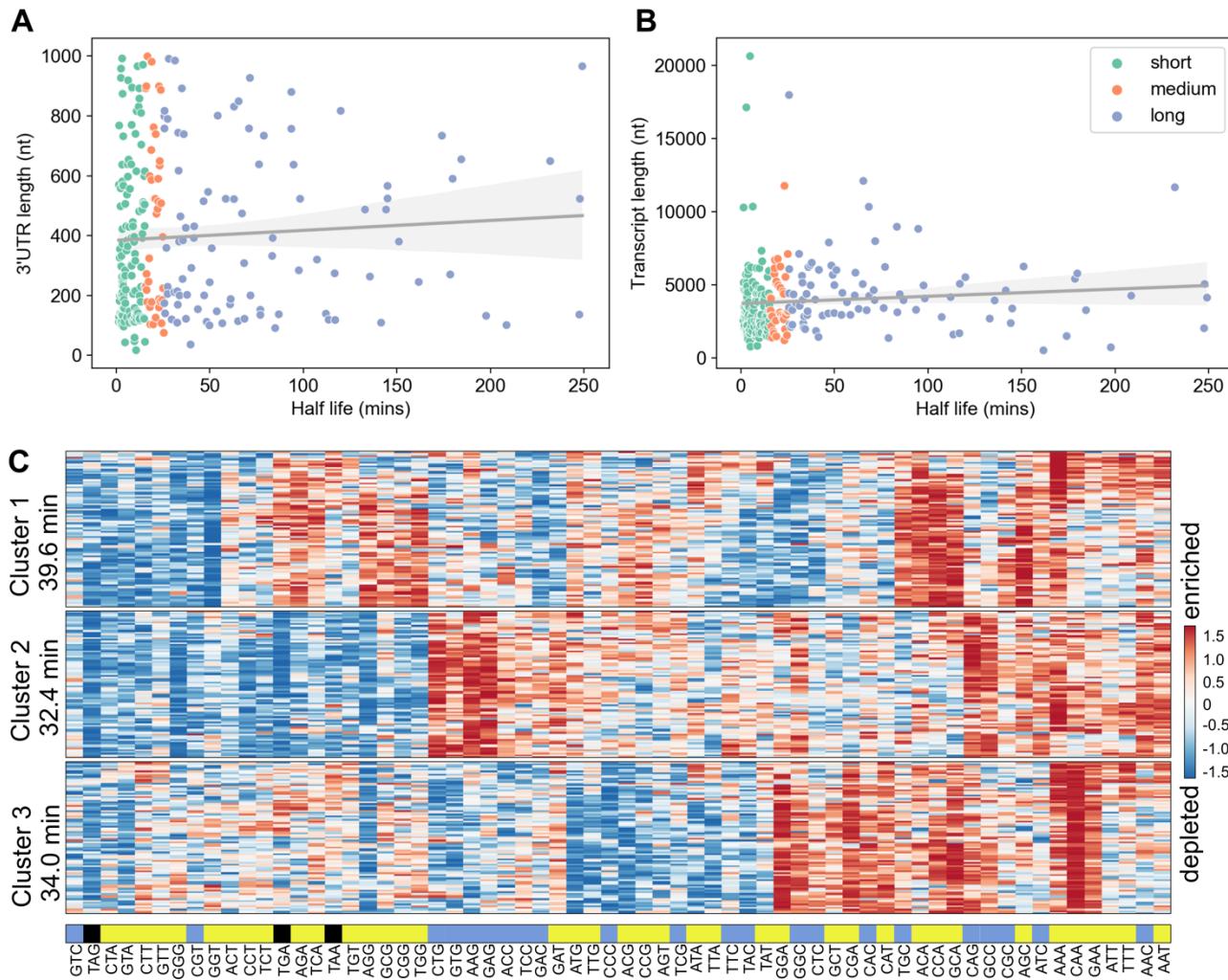
**Figure S4: High embryo to embryo variation in transcript numbers masks variation due to degradation over cell division.** (A) Parameter estimates for degradation rates and credible regions of short and long half-life genes shown in Figure 2D. (B) RNA-seq data for *gogo* fitted with the Gaussian process model. A half-life of 19 minutes is inferred for this gene. (C) Confocal images of pre- and post-division during the 13th and 14th nuclear cycles showing nuclei stained with DAPI (blue) and single mRNAs (white). (D) Data for the number of mRNAs per cell for embryos at various time points during the cell division, fitted with an exponential function from which a half-life of 30 min was calculated. The data has a low signal to noise ratio of 0.0013, meaning that the reduction in transcript numbers over time due to degradation (signal) is much smaller than the natural embryo to embryo variation in transcript numbers (noise). (E) Theoretical data demonstrating the reduction in transcript numbers that would be expected for a mRNA with a 19 min half-life over a 210 second time frame, which is a reduction of ~10%.





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1125 **Figure S6: Sub-clustering of zygotic transcripts with intronic data in intronic cluster 2 using**  
1126 **GPclust.** Data for all mRNAs in the cluster are shown with shaded credible regions and inferred function  
1127 as a solid line for intronic cluster 2. The number of mRNAs in each cluster is shown in the top right  
1128 corner of each plot and graphs are arbitrarily coloured. The mean half-lives of the transcripts in the  
1129 cluster are shown where the cluster has >2 transcripts with estimated half-lives.  
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1132 **Figure S7: mRNA properties and stability.** (A) Graph showing the correlation between mRNA half-  
1133 life and 3' UTR length. Data fit with linear regression model, Pearson's  $r = 0.06$ ,  $p = 0.34$ . (B) Correlation  
1134 between mRNA total length and half-life. Transcripts are coloured by half-life category short (green),  
1135 medium (orange) or long (blue). Data fit with linear regression model, Pearson's  $r = 0.09$ ,  $p = 0.13$ . (C)  
1136 Transcripts were clustered based on similar codon usage using K-means and the level of enrichment of  
1137 each codon within the transcript is shown in the heatmap. The average half-life of each cluster is shown  
1138 to the left. Codons are coloured by whether they are optimal (blue) vs non-optimal (yellow) with stop  
1139 codons shown in black.

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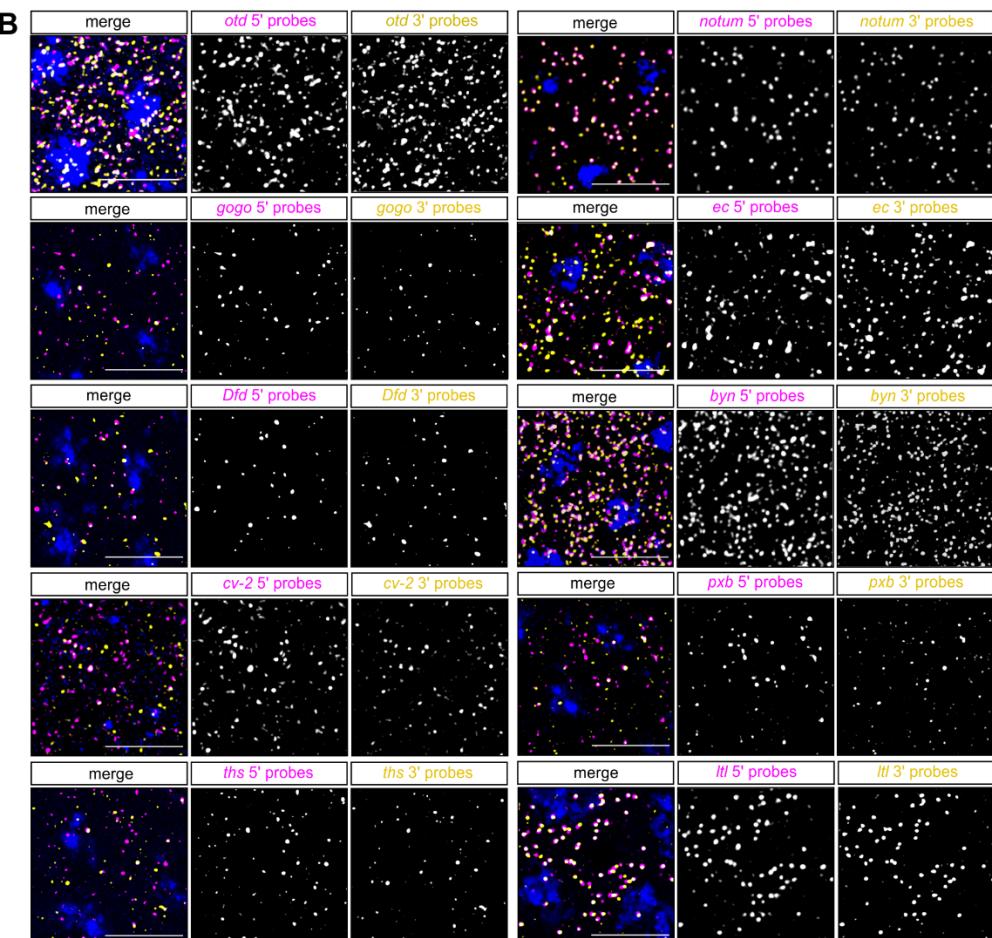
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**A**

Transcript half-lives used in smFISH experiments

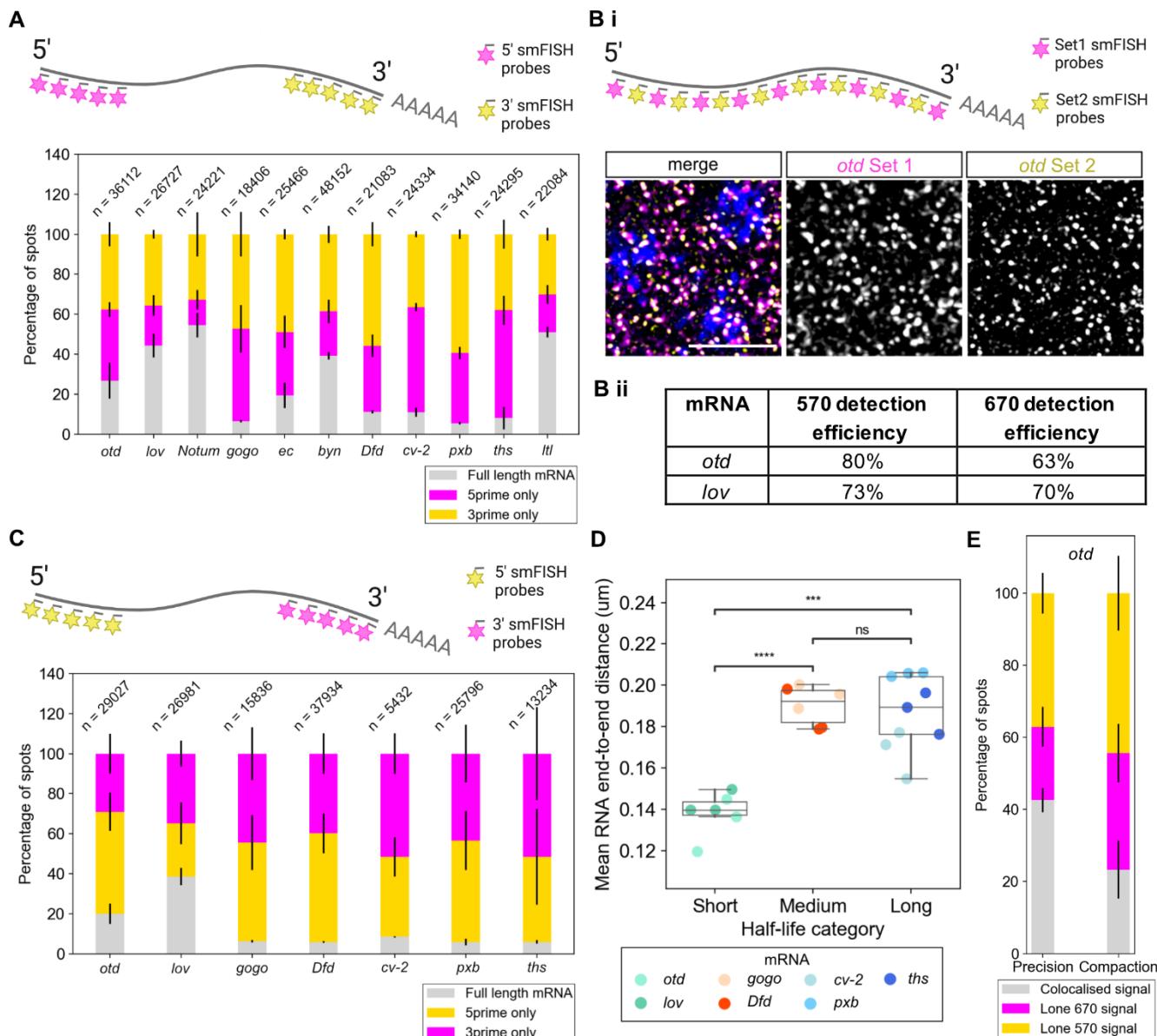
Gene	Transcript ID	Half-life	Category
<i>otd</i>	FBtr0331305	3	short
<i>Neu2</i>	FBtr0332718	5	short
<i>iov</i>	FBtr0072458/FBtr0330231	9/9	short
<i>Notum</i>	FBtr0075435	12	short
<i>gogo</i>	FBtr0074848	19	medium
<i>ec</i>	FBtr0112802/FBtr0070606	18/20	medium
<i>Dfd</i>	FBtr0081621	22	medium
<i>byn</i>	FBtr0332071/FBtr0332070	22/23	medium
<i>cv-2</i>	FBtr0071610	28	long
<i>pxb</i>	FBtr0083162	47	long
<i>ths</i>	FBtr0088030	54	long
<i>Itl</i>	FBtr0332144	249	long

**B**



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1143 **Figure S8: Compaction of mRNAs in early embryos.** (A) A table of the transcripts used in smFISH  
1144 experiments. (B) Confocal images of fixed embryos showing smFISH detection of the 5' (magenta) and  
1145 3' (yellow) ends of the mRNAs used in the compaction experiments quantitated in Figure 4F.  
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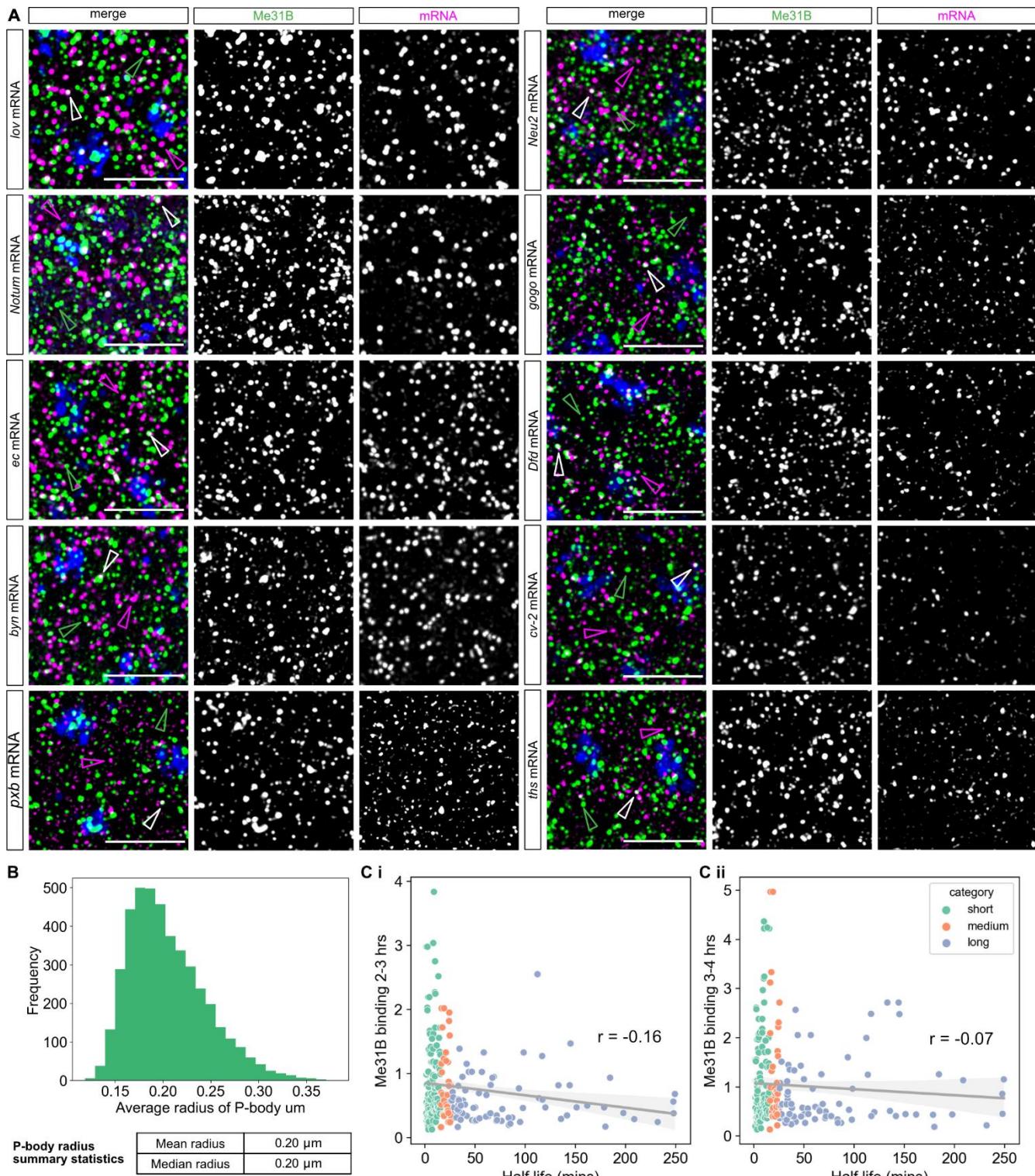


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**Figure S9: Control experiments for smFISH.** (A) Proportions of 5' (670) and 3' (570) ends and whole mRNAs detected in smFISH experiments for all the compaction forward data described in Figure 4D-F. Numbers represent total mRNAs in image (whole, 5' only and 3' only). Schematic illustrates the forward staining scheme. (B) (i) Schematic shows full length *otd* mRNAs detected using probes with alternating labels used as a precision control. Confocal images of fixed embryos stained with alternating smFISH probes. All images are maximum projections of 7 Z slices. Scale bars: 5  $\mu$ m. (ii) Table of detection efficiencies for the two mRNAs used in precision control experiments. (C) Proportions of 5' (570) and 3' (670) ends and whole mRNAs detected in smFISH experiments for reverse data (with switched fluorophores). Numbers represent total mRNAs in image as in A. (D) Graph shows the end-to-end distances of mRNAs with different stabilities in the reverse fluorophore experiment to Figure 4D, data are shown in boxplots for each half-life category with n = 3 embryos for each transcript. (E) Proportion of *otd* signals detected in either the 570, 670 or colocalised signal in the precision or both compaction experiments.

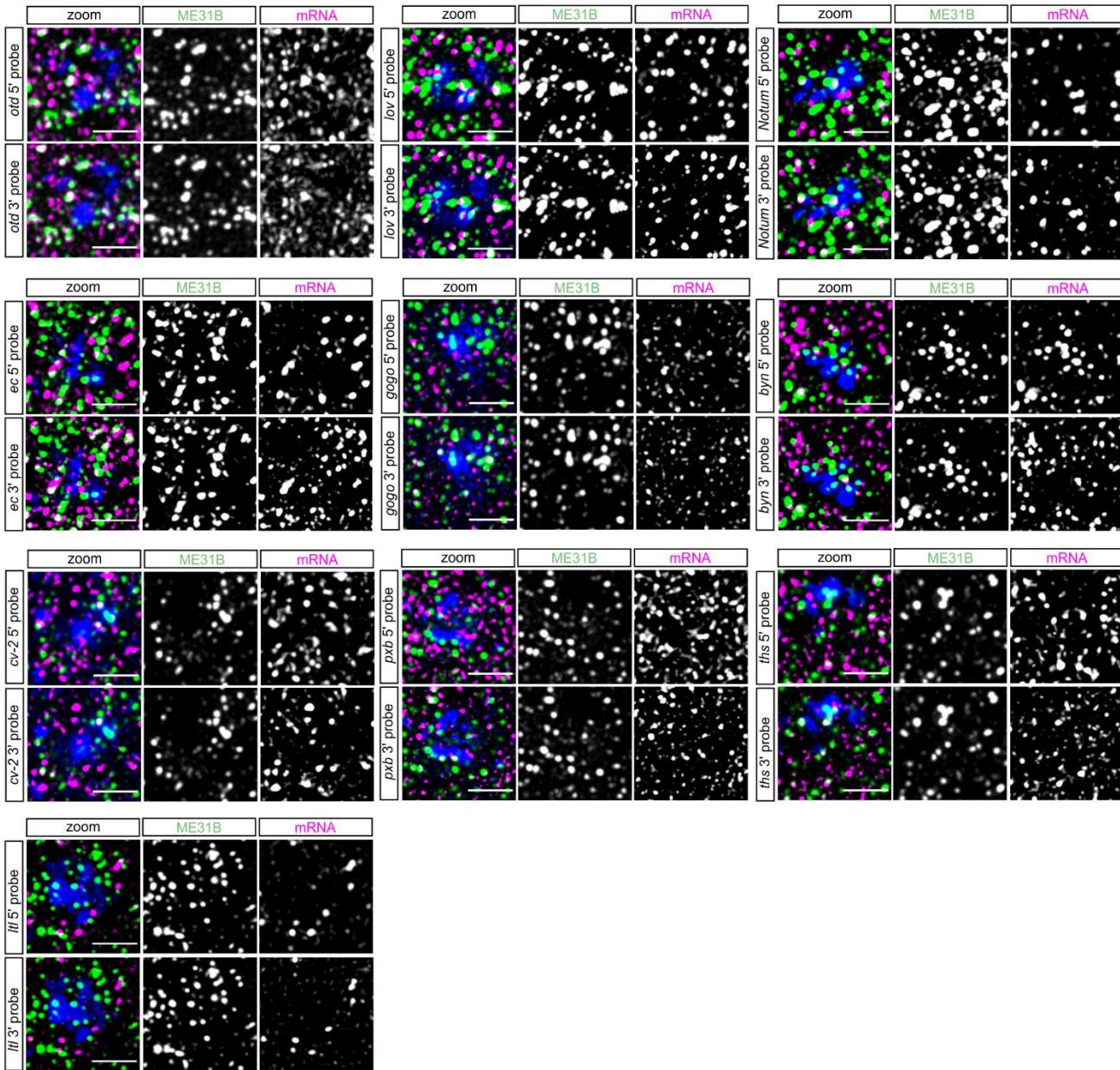
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1164 **Figure S10: Confocal images of mRNAs with various stabilities and their colocalisation with the**  
1165 **P-body marker Me31B.** (A) Confocal images of early nc14 Me31B-GFP embryos showing smFISH  
1166 staining of the indicated test mRNAs. DAPI marks the nuclei (blue), mRNAs are shown in magenta  
1167 (magenta arrowhead), GFP-Me31B marking P-bodies in green (green arrowhead) and mRNAs

1168 colocalising with P-bodies in white (white arrowhead). All images are MIP of 7 Z slices of a confocal  
1169 image. Scale bar: 5 $\mu$ m (B) Analysis of P-body sizes reveals an average radius of 0.2  $\mu$ m. (C) Analysis  
1170 of correlation between Me31B binding from (i) 2-3 hour embryos and (ii) 3-4 hr embryos with model half-  
1171 life. A significant negative correlation ( $r = -0.16$ ,  $p = 0.014$ ) is found at 2-3 hours but no significant  
1172 relationship is found at 3-4 hours ( $r = -0.07$ ,  $p = 0.30$ ).



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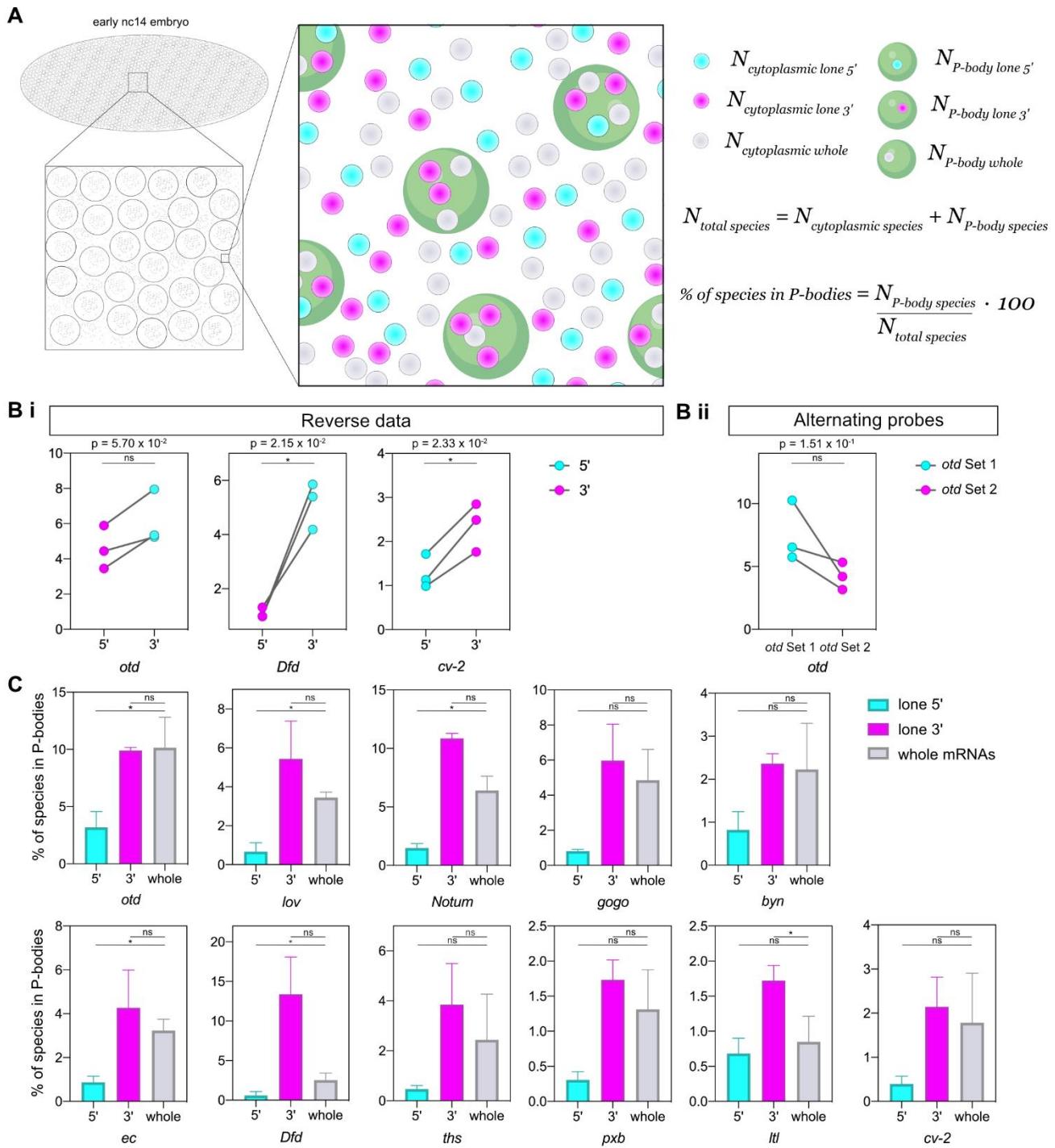
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**Figure S11: Confocal images of mRNAs and their 5' and 3' end colocalisation with the P-body marker Me31B.** Confocal images of early nc14 Me31B-GFP embryos showing smFISH staining of the indicated test mRNAs with 5' and 3' probe sets. For each test mRNA, the same region of the embryo is shown with the 5' and 3' mRNA probe sets separately for clarity. mRNAs are shown in magenta, GFP-Me31B marking P-bodies in green and DAPI labelling nuclei in blue. All images are MIP of 7 Z slices of a confocal image. Scale bar: 2μm.



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1182 **Figure S12: Proportions of lone 5', lone 3' and whole mRNAs colocalised with P-bodies.** (A) Schematic demonstrating quantitation of the proportion of each species in P-bodies. 5' and 3' spots are detected and then paired (see methods) to give 3 species - whole mRNAs (grey), lone 5' ends (cyan) and lone 3' ends (magenta). The number of each species that colocalises with P-bodies is divided by the total number of that species to give a percentage enrichment in P-bodies. (Bi) Quantification of the percentage of unpaired mRNA 5' and 3' ends in P-bodies relative to the total number of lone 5' or 3' ends in the switched probe fluorophore experiments for *otd*, *Dfd* and *cv-2* (see also Figure 6C). Paired t-test used to determine significance. (Bii) (C) Percentages of each species (whole, lone 5' and lone 3')

1190 in P-bodies across the test set of mRNAs. mRNAs are ordered by their half-life from the shortest (*otd*)  
1191 to the longest (*cv-2*) half-life. One-way repeated measures ANOVA used to determine significance with  
1192  $\alpha = 0.05$ .

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