

## Pseudouridine-dependent ribosome biogenesis regulates translation of polyglutamine proteins during *Drosophila* oogenesis

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## Abstract

Stem cells in many systems, including *Drosophila* germline stem cells (GSCs), increase ribosome biogenesis and translation during terminal differentiation. Here, we show that pseudouridylation of ribosomal RNA (rRNA) mediated by the H/ACA box is required for ribosome biogenesis and oocyte specification. Reducing ribosome levels during differentiation decreased the translation of a subset of mRNAs that are enriched for CAG repeats and encode polyglutamine-containing proteins, including differentiation factors such as RNA-binding Fox protein 1. Moreover, ribosomes were enriched at CAG repeats within transcripts during oogenesis. Increasing TOR activity to elevate ribosome levels in H/ACA box-depleted germlines suppressed the GSC differentiation defects, whereas germlines treated with the TOR inhibitor rapamycin had reduced levels of polyglutamine-containing proteins. Thus, ribosome biogenesis and ribosome levels can control stem cell differentiation via selective translation of CAG repeat-containing transcripts.

45 **Introduction**

46 Understanding how stem cells self-renew and differentiate is crucial to understanding the  
47 mechanisms of development and disease (Cinalli et al., 2008; Morrison et al., 1997; Tang, 2012).  
48 Defects in ribosome biogenesis can impair stem cell differentiation and lead to diseases  
49 collectively called ribosomopathies (Armistead and Triggs-Raine, 2014; Barlow et al., 2010;  
50 Brooks et al., 2014; Calo et al., 2018; Higa-Nakamine et al., 2012; Mills and Green, 2017). Protein  
51 synthesis often increases during stem cell differentiation (Sanchez et al., 2016; Teixeira and  
52 Lehmann, 2019; Zhang et al., 2014), and inhibiting translation by modulating Target of Rapamycin  
53 (TOR) activity blocks terminal differentiation of various stem cells (Martin et al., 2022; Neumüller  
54 et al., 2008; Sanchez et al., 2016; Sun et al., 2010; Zhang et al., 2014). Nevertheless, how  
55 ribosome levels and translation control differentiation remains incompletely understood. In the  
56 ribosomopathy Diamond-Blackfan anemia (DBA), mutations in ribosomal proteins limit the pool  
57 of available ribosomes, which alters the translation of a select subset of transcripts in  
58 hematopoietic stem and progenitor cells, leading to impaired erythroid lineage commitment  
59 (Khajuria et al., 2018; Xue and Barna, 2012).

60

61 RNAs are extensively modified by post-transcriptional modifications (PTMs), including  
62 pseudouridylation (Granneman, 2004; Sloan et al., 2017; Tafforeau et al., 2013; Watkins and  
63 Bohnsack, 2012). The ribosomal RNA (rRNA) pseudouridine synthase subunit DKC1 is mutated  
64 in the ribosomopathy X-linked dyskeratosis congenita (X-DC), an inherited bone marrow failure  
65 syndrome that is sometimes associated with impaired neurodevelopment (Knight et al., 1999).  
66 DKC1 is a member of the snoRNA-guided H/ACA box, which deposits pseudouridine on rRNA at  
67 functionally important sites of the ribosome (Charette and Gray, 2000; Czekay and Kothe, 2021;  
68 Penzo and Montanaro, 2018). Mutations in DKC1 (Nop60B in *Drosophila*) can impair ribosomal  
69 binding to tRNAs and to internal ribosomal entry sites (IRES) from yeast to humans (Jack et al.,  
70 2011). Nevertheless, how H/ACA box dysfunction generates tissue-specific defects remains  
71 unclear.

72

73 During *Drosophila* oogenesis, differentiation of germline stem cells (GSCs) to an oocyte is  
74 sensitive to both ribosome biogenesis and translation (Blatt et al., 2020; Cinalli et al., 2008; Martin  
75 et al., 2022; Sanchez et al., 2016; Zhang et al., 2014). Oogenesis occurs in ovarioles beginning  
76 with the germline stem cells (GSCs) in the germaria (**Figure 1A**) (Lehmann, 2012; Xie and  
77 Spradling, 2000). The GSCs undergo asymmetric cell division to self-renew and give rise to  
78 daughter cells called cystoblasts (CBs) (Chen and McKearin, 2003b; Ohlstein and McKearin,  
79 1997). The CB differentiates undergoing four incomplete mitotic divisions giving rise successively  
80 to 2-, 4-, 8-, and finally 16-cell cysts (Spradling et al., 1997; Xie and Spradling, 1998, 1998). One  
81 cell in the 16-cell cyst stage becomes the oocyte while the remaining 15 cells become the nurse  
82 cells that support the growing oocyte (**Figure 1A**) (Huynh and St Johnston, 2004; Kugler and  
83 Lasko, 2009; Lantz et al., 1994; Navarro et al., 2004). The GSCs and the CBs are marked by a  
84 round cytoskeletal structure called the spectrosome while the cysts are marked by a branched  
85 structure called the fusome (Chen and McKearin, 2003a; Ting, 2013). The 16-cell cyst becomes  
86 encapsulated by somatic cells to create an egg chamber that then goes through progressive  
87 development producing a mature egg (**Figure 1A**) (Huynh and St Johnston, 2004; Koch et al.,  
88 1967; Navarro et al., 2004).

89  
90 In the CB, expression of Bag of marbles (Bam) promotes the progression from CB to an 8-cell  
91 cyst stage where expression of RNA-binding Fox protein 1 (Rbfox1) and Bruno 1 (Bru1) are  
92 required to specify an oocyte (Carreira-Rosario et al., 2016; Sugimura and Lilly, 2006). In parallel,  
93 several cells in the cysts initiate recombination mediated by the synaptonemal complex, which  
94 includes proteins such as crossover suppressor on 3 of Gowen (c(3)G), but only the specified  
95 oocyte commits to meiosis (Collins et al., 2014; Hughes et al., 2018; Page and Hawley, 2001).  
96 Rbfox1 is not only critical for female fertility but also for neurological functions (Carreira-Rosario  
97 et al., 2016; Gehman et al., 2012, 2011; Kucherenko and Shcherbata, 2018). Why some  
98 transcripts encoding differentiation factors, such as Rbfox1, are sensitive to ribosome levels is  
99 not known.

100  
101 **Results**

### 102 **RNA modifications are dynamic and essential for oogenesis**

103 We aimed to identify dynamic RNA PTMs during oogenesis. Therefore, we enriched for five  
104 stages of oogenesis (1. GSCs; 2. GSC Daughter/CBs; 3. early-cysts; 4. germaria and early-stage  
105 egg chambers (young wild type (YWT)); 5. and late-stage egg chambers (WT)) which are critical  
106 milestones of germline development (Flora et al., 2018; McKearin and Spradling, 1990; Ohlstein  
107 and McKearin, 1997; Xie and Spradling, 1998). We performed tandem mass spectrometry on total  
108 RNA extracted from each of the enriched stages (**S1A-S1A'''**) (Flora et al., 2018; McKearin and  
109 Spradling, 1990; Ohlstein and McKearin, 1997; Xie and Spradling, 1998). For each enriched  
110 developmental stage, we performed 5 biological replicates, each with 3 technical replicates. We  
111 identified 18 groups of RNA PTMs represented by distinct mass:charge ratios, composed of 42  
112 distinct RNA PTMs from a total of 172 known PTMs (**Figure 1B, Supplementary Table 1**).  
113 Pseudouridine, which is the most frequent PTM in RNA (Durairaj and Limbach, 2008), was the  
114 most abundant modification at all stages, followed by the monomethylations of the canonical RNA  
115 bases (**Figure 1B, Supplementary Table 1**). Furthermore, we discovered a cohort of RNA PTMs,  
116 including inosine and dihydrouridine, that were not previously described during oogenesis (**Figure**  
117 **1B, Supplementary Table 1**). Most RNA PTMs, including pseudouridine, were dynamic during  
118 GSC differentiation into an oocyte (**Figure 1B, Supplementary Table 1**).

119  
120 To determine if the RNA modifications play a role in germline development, we performed an RNA  
121 interference (RNAi) screen utilizing a germline-specific *nanos-GAL4* driver to deplete RNA  
122 modifying enzymes in the germline, followed by immunostaining for Vasa, a germline marker, and  
123 1B1, a marker of the cell membranes, spectrosomes and fusomes (Lasko and Ashburner, 1988;  
124 Zaccai and Lipshitz, 1996). We screened 33 unique genes annotated and predicted to be involved  
125 in RNA modification, and based on availability additional independent RNAi lines, for a total of 48  
126 lines. Of the 33 distinct gene knockdowns, 2 resulted in loss of the germline, 14 in germaria  
127 defects, and 3 in egg chamber defects (**Supplementary Table 2**).

128  
129 **The pseudouridine-depositing H/ACA box is required for oocyte specification**  
130 Among the genes whose knockdown that caused defects in germaria, we found all four encoding  
131 components of the rRNA pseudouridine synthase, the H/ACA box: the catalytic subunit Nucleolar  
132 protein at 60B (Nop60B) and complex members CG7637 (Nop10), CG4038 (Gar1) and NHP2

133 (**Figure 1C, Supplementary Table 2**) (Giordano et al., 1999; Ni et al., 1997). Germline depletion  
134 of Nop60B in the background of an endogenous, GFP-tagged Nop60B reporter led to significantly  
135 reduced GFP levels in the germline (**Figure S1B-S1D**) (Sarov et al., 2016), verifying knockdown  
136 of *nop60B*. In addition, RT-qPCR analysis revealed significantly reduced levels of *Nop10* and  
137 *Nop60B* mRNAs upon germline knockdown (**Figure S1E**). Depletion of the H/ACA box  
138 components did not result in a germline viability defect, but rather to specific loss of GSCs and a  
139 cyst differentiation defect. Specifically, transition from 8-cell cyst stage to an egg chamber was  
140 blocked, as measured by the accumulation of 8-cell cysts (**Figure 1D-1J, S1F-S1P,**  
141 **Supplementary Table 2**) (Morita et al., 2018; Sanchez et al., 2016), which led to an absence of  
142 egg chambers and, in turn, sterility (**Figure S1Q**). Thus, the H/ACA box is required in the female  
143 germline for proper cyst differentiation.

144  
145 We further investigated the role of the H/ACA box in cyst differentiation by analyzing control and  
146 H/ACA box germline-depleted ovaries carrying the differentiation reporter, BamGFP. We also  
147 stained ovaries for Vasa, 1B1, and the cyst-differentiation factors, Rbfox1 or Bru1 (Carreira-  
148 Rosario et al., 2016; Chen and McKearin, 2003b; Sugimura and Lilly, 2006). We found that cysts  
149 lacking H/ACA box members express BamGFP but have significantly reduced levels of Rbfox1  
150 and Bru1 (**Figure 2A-G, S2A-S2D**). Moreover, cysts lacking H/ACA box components did not  
151 specify an oocyte, as cysts were devoid of localized Egalitarian (Egl), the oocyte determinant, and  
152 exhibited reduced expression of the synaptonemal complex component C(3)G (**Figure S2E-S2L**)  
153 (Anderson et al., 2005; Carpenter, 1994; Huynh and St Johnston, 2000; Mach and Lehmann,  
154 1997; Page and Hawley, 2001), consistent with a differentiation block.

155  
156 To determine the specific stage of oogenesis that requires H/ACA box activity, we first  
157 characterized the expression of the endogenous, GFP-tagged Nop60B reporter (Sarov et al.,  
158 2016). Nop60B-GFP levels increased from the cyst stages to early egg chambers (**Figure S3A-**  
159 **S3B**). Utilizing a pseudouridine antibody, we observed a corresponding increase in pseudouridine  
160 levels from the 8-cell cyst to the newest egg chamber and this increase depended on the H/ACA  
161 box (**Figure S3C-S3G**). Given these observations, and that loss of H/ACA box components  
162 resulted in an accumulation of 8-cell cysts (**Figure S1P**) (Morita et al., 2018), we hypothesized  
163 that the H/ACA box is required in the cysts for the transition into an oocyte. To test this, we  
164 depleted *Nop60B* and *Nop10* in the cysts utilizing a *bamGAL4* driver, which is active in the 2-8  
165 cell cyst stages. We observed an accumulation of cysts with significantly reduced levels of Rbfox1  
166 (**Figure S4A-S4H**) (Carreira-Rosario et al., 2016; Chen and McKearin, 2003b). Taken together,  
167 these data suggest that the H/ACA box is required in the cyst stages for differentiation into an  
168 oocyte.

169  
170 **The H/ACA box promotes ribosome biogenesis and the translation of differentiation**  
171 **factors during oogenesis**

172 The primary activity of the H/ACA box is to deposit pseudouridine on rRNA, thereby promoting  
173 ribosome biogenesis in the nucleolus (Gilbert, 2011; Kiss et al., 2010; Ni et al., 1997; Omer et al.,  
174 2000). Nop60B-GFP colocalized with Fibrillarin in the nucleolus as previously observed (Ochs et  
175 al., 1985) (**Figure S5A-S5B**). In addition, loss of *Nop10* and *Nop60B* resulted in cysts with  
176 hypertrophic nucleoli compared to wild-type cysts, suggesting a ribosome biogenesis defect

177 (Figure S5C-S5F) (Neumüller et al., 2008; Sanchez et al., 2016). To verify that the H/ACA box  
178 deposits pseudouridine on rRNA during oogenesis, we co-immunopurified the 40S and 60S  
179 ribosomal subunits from the germline, utilizing a germline-enriched HA-tagged ribosomal protein  
180 RpS5b (Figure S5G-S5I) (Jang et al., 2021) (Chen and Dickman, 2017). Mass spectrometry  
181 analysis showed that loss of the H/ACA box member Nop10 led to a significant decrease of  
182 pseudouridine on rRNA relative to controls (Figure 3A, Supplemental Table 3). In addition, we  
183 observed a decrease in both the 40S and 60S subunits and in polysomes of Nop60B-depleted  
184 ovaries as compared to controls (Figure S5J) (Cheng et al., 2019), suggesting a ribosome  
185 biogenesis defect upon loss of the H/ACA box. Thus, consistent with previous findings, the H/ACA  
186 box deposits pseudouridine on rRNA to promote ribosome biogenesis in the germline.  
187

188 To test if loss of the H/ACA box and consequent aberrant ribosome biogenesis affects mRNA  
189 translation during oogenesis, we performed polysome-seq of ovaries depleted of Nop60B in the  
190 germline and of gonads enriched for cysts stages (Flora et al., 2018; Martin et al., 2022; Ohlstein  
191 and McKearin, 1997). As enriching for early cyst stages includes a heat shock step (see methods),  
192 we also analyzed early-stage egg chambers to control for heat shock effects. We detected 465  
193 mRNAs with a reduced polysome association in *Nop60B*-knockdown versus the controls,  
194 whereas 638 mRNAs showed an increased polysome association (Figure 3B, S6A-S6C). These  
195 data suggest that the H/ACA box regulates the synthesis of a cohort of proteins. GO term analysis  
196 revealed that mRNAs with an elevated polysome association were enriched in the mitotic cell  
197 cycle, whereas those with reduced polysome association included factors that promote meiosis  
198 1, meiotic cell cycle and homologous chromosome segregation (Figure 3C, S6D), such as the  
199 synaptonemal complex members C(3)G and Corona (conA), consistent with reduced C(3)G  
200 protein levels upon depletion of the H/ACA box (Figure S2I-S2L).  
201

202 The levels of *Rbfox1* and *Bru1* mRNAs were not significantly reduced in the germline upon  
203 depletion of the H/ACA box, as indicated by fluorescent *in situ* hybridization (Figure 3D-3G,  
204 Figure S6E-S6H). To determine if the H/ACA box is required for translation of *Rbfox1* and *Bru1*,  
205 we overexpressed *Rbfox1* or *Bru1* under the control of UAS/GAL4 system. *Rbfox1* and *Bru1*  
206 proteins were detected in the control germlaria, but not in the H/ACA box-depleted germlaria  
207 (Figure 3H-3J, S6I-S6K) (Carreira-Rosario et al., 2016; Filardo and Ephrussi, 2003), suggesting  
208 that their translation is impaired upon loss of the H/ACA box.  
209

210 We considered that the H/ACA box is required for oogenesis due to its role in ribosome  
211 biogenesis. This hypothesis predicts that compromised ribosome biogenesis will phenocopy loss  
212 of H/ACA box components. We impaired ribosome biogenesis by depleting ribosomal protein  
213 paralogs RpS10b and RpS19b in the germline, as the depletion of other ribosomal proteins that  
214 do not have paralogs results in GSC differentiation defects or loss of cyst stages that would mask  
215 the cyst differentiation block (Jang et al., 2021; Martin et al., 2021; McCarthy et al., 2022; Sanchez  
216 et al., 2016). Depletion of RpS10b and RpS19b phenocopied loss of H/ACA box components,  
217 leading to a block in cyst differentiation and decreased levels of *Rbfox1* and *Bru1* proteins without  
218 a concomitant loss of their mRNAs (Figure S7A-S7K, S8A-S8H). The H/ACA box can also  
219 pseudouridylate mRNAs and tRNAs (Czekay and Kothe, 2021). However, immunopurification of  
220 pseudouridine did not enrich for the mRNAs with perturbed translation upon loss of the H/ACA

221 box (**Supplemental Table 4**), suggesting that these targets are not directly pseudouridylated. In  
222 addition, whereas the loss of the H/ACA box blocks cyst differentiation, loss of tRNA  
223 pseudouridylation enzymes results in a different phenotype – loss of cyst stages (**Figure S9A-S9J**). Thus, our data suggest that the H/ACA box and pseudouridinylation are important for  
224 ribosome biogenesis during oogenesis.  
225

226  
227 **H/ACA box-dependent differentiation factors are PolyQ proteins**  
228 To identify shared properties among the mRNAs with reduced polysome association upon loss of  
229 the H/ACA box, we performed a motif analysis of the 5'UTRs, CDS and 3'UTRs of this subset of  
230 mRNAs compared to a set of control mRNAs. We observed a motif of repeating CAG nucleotides  
231 that was highly enriched in the CDS of downregulated transcripts compared to the control  
232 unregulated mRNAs (**Figure 4A**). In addition, we found motifs that were enriched in the 3'UTRs or  
233 5'UTRs of downregulated transcripts, albeit in a smaller subset of RNAs  
234 (**Supplementary Table 5**). The CAG motifs in the downregulated transcripts were in frame, such  
235 that the encoded proteins are highly enriched in glutamine (Q) over a region of 21 amino acids  
236 (**Figure 4B**). Indeed, Rbfox1 and Bru1 both contain such repeating CAG motifs in the mRNA and  
237 polyQ in the protein (**Figure S10A-S10D**) (Kucherenko and Shcherbata, 2018).  
238

239 To determine if the H/ACA box is required to translate CAG repeat-containing mRNAs during  
240 oogenesis, we expressed a CAG reporter encoding an HA-tagged, polyQ protein, that was  
241 previously used to model polyglutamine toxicity from Huntington's disease (**Figure 4C**) (Fayazi et  
242 al., 2006). We co-expressed the CAG reporter with GFP in the control, to ensure equal GAL4  
243 dosage, and the CAG reporter in Nop10-depleted germlines. We found that loss of the H/ACA  
244 box specifically reduced the levels of the polyQ protein (**Figure 4D-4F, S10E-S10F**). Furthermore,  
245 depletion of RpS19b and Nop60B also resulted in a significant decrease in polyQ protein  
246 accumulation, but loss of Nop60B did not significantly alter the levels of GFP (**Figure S10G-S10M**). Thus, the H/ACA box and ribosome biogenesis are required for translation of polyQ-containing  
247 proteins.  
248

249  
250 **CAG repeat regions show increased density of ribosomes**  
251 One proposed mechanism of polyQ expansion-induced defects is the disruption of translation by  
252 ribosome stalling (Eshraghi et al., 2021). To determine if the H/ACA box affects elongating  
253 ribosomes on, and hence translation of, mRNAs encoding polyQ proteins, we performed ribosome  
254 footprinting (Ribo-Seq). Because we were unable to acquire sufficient material from H/ACA box-  
255 depleted germlines, we utilized ovaries enriched for late-stage oocytes (late-ovaries), which have  
256 reduced pseudouridine levels but can be obtained in sufficient quantity (**Figure 1B, Supplementary Table 1**). Three late-ovary Ribo-Seq libraries were generated, each with a corresponding RNA-Seq library. Correlation analysis showed consistent and reproducible  
257 ribosome footprint distributions among the three Ribo-Seq libraries (Pearson  $r > 0.9$  for all  
258 comparisons; **Supplemental Table 6**). We hypothesized that stalled ribosomes might result in  
259 local enrichment of ribosome footprints. We therefore sought to identify peaks in the Ribo-Seq  
260 data across the transcriptome. Our peak detection and subsequent motif analysis identified 123  
261 mRNAs containing at least one CAG-rich segment within 30 nucleotides of a ribosome footprint  
262 peak in at least 2 of the 3 Ribo-Seq libraries that were not present in the RNA-seq libraries (**Figure**  
263  
264

265 **4G, Supplemental Table 6).** 46 of the 123 identified mRNAs encode at least one polyQ tract ( $\geq$   
266 4 consecutive CAG codons) near ribosome footprint peaks (**Figure 4H, Supplemental Table 6**).  
267 This motif is highly reminiscent of the motif identified in the mRNAs with low polysome association  
268 in H/ACA box depleted germlines (**Figure 4A**).  
269

270 The motif identified by Ribo-Seq contains 5 CAGs in a row (**Figure 4A and 4G**). To determine if  
271 the 5-CAG motif is overrepresented in the set of target mRNAs with low polysome association,  
272 we performed a Find individual Motif Occurrence (FIMO) analysis. We found that 181 out of 465  
273 (39%) targets contain a significant motif representative of the 5-CAG motif including *bru1* and  
274 *C(3)G* (**Figure S2A-S2D, S2I-S2K, Supplemental Table 7**). We infer that CAG repeat sequences  
275 have high ribosome density and are present in mRNAs whose translation is sensitive to reduced  
276 ribosome biogenesis during oogenesis.  
277

### 278 **Tor signaling partially restores differentiation and modulates polyQ translation**

279 The Target of Rapamycin (TOR) pathway is a critical positive regulator of ribosome biogenesis  
280 (Wullschleger et al., 2006; Yerlikaya et al., 2016). To further determine if loss of the H/ACA box  
281 blocks cyst differentiation due to reduced ribosome biogenesis, we increased ribosome  
282 biogenesis by overexpressing the TORC1 co-factor, Raptor, in the H/ACA box-depleted germline  
283 (Martin et al., 2006; Wang et al., 2012). We observed a partial yet significant alleviation of the cyst  
284 differentiation defect, such that an egg chamber was formed (**Figure 4I-4J**). We next asked if  
285 decreasing TOR activity and, in turn, ribosome biogenesis could diminish expression of the polyQ  
286 reporter. Specifically, flies expressing the germline CAG reporter were treated with the inhibitor  
287 of mTOR, Rapamycin, and displayed lower levels of germline polyQ when compared to controls  
288 (**Figure S10N-S10P**). Thus, our data suggest that modulating ribosome levels via the Tor pathway  
289 can effectively regulate translation of polyQ-containing proteins.  
290

### 291 **Discussion**

292 We used the power of *Drosophila* genetics combination with mass spectrometry to determine the  
293 developmental profile of RNA PTMs and identify a cohort of PTMs that are required for proper  
294 oogenesis. Specifically, we found that pseudouridine abundance is dynamic and regulated by the  
295 H/ACA box, a pseudouridine synthase, and is required for proper cyst differentiation and oocyte  
296 specification. Using polysome-seq analysis, we found that CAG repeat mRNAs encoding polyQ-  
297 containing proteins have reduced polysome association upon loss of the H/ACA box. These polyQ  
298 proteins include germ cell differentiation and meiosis promoting factors such as *Rbfox1* and *Bru1*.  
299 Moreover, we found that CAG repeat regions accumulate ribosomes, potentially acting as a  
300 ribosome sink. Taken together, our data suggest under condition of low ribosome levels, the CAG  
301 repeat containing regions can impede proper translation by sequestering ribosomes internally  
302 causing translation of polyQ-containing proteins to be sensitive to ribosome levels (**Figure 4K**).  
303 Taken together, we find that the H/ACA box promotes ribosome biogenesis during oogenesis and,  
304 in turn, the translation of CAG repeat mRNAs required for differentiation (**Figure 4K**).  
305

306 Ribosomopathies predispose individuals to neurological deficits, but the etiology of this defect is  
307 unclear (Aspesi and Ellis, 2019; Cheng et al., 2019; Mills and Green, 2017). Neurons express and  
308 require several polyQ-containing proteins, including *Rbfox1* (Gehman et al., 2012, 2011;

309 Kucherenko and Shcherbata, 2018). We find that the translation and levels of Rbfox1 are sensitive  
310 to ribosome levels during oogenesis (McCarthy et al., 2022). By extension, neuronal deficits  
311 observed in ribosomopathies could be due to inability to translate critical polyQ-containing  
312 proteins in neurons.

313  
314 While polyQ stretches facilitate phase transition, large CAG expansion and polyQ protein  
315 aggregates are associated with diseases such as Huntington's disease (Adegbuyiro et al., 2017;  
316 Sugars and Rubinsztein, 2003). A genome-wide association study revealed that the onset of  
317 Huntington's disease is due to large expansions of CAG repeats and is accelerated by DNA repair  
318 genes as well as E3 ubiquitin protein ligase (UBR5) (Lee et al., 2019). In embryonic stem cells,  
319 UBR5 has been shown to physically interact with the H/ACA box to promote rRNA maturation  
320 suggesting that these factors could collaborate to contribute to early onset of Huntington's  
321 Disease (Saez et al., 2020). Furthermore, Huntington's Disease mouse models have shown CAG  
322 expansions induce ribosome stalling by impeding ribosome translocation, thereby inhibiting  
323 protein synthesis (Eshraghi et al., 2021). These data together with our findings that, during  
324 development, the H/ACA box promotes translation of CAG repeat containing RNAs suggest that  
325 translation dysregulation could be a key feature of CAG expansion diseases. While the early onset  
326 is determined by CAG length, translation of CAG repeats into polyQ proteins can cause protein  
327 aggregation and toxicity (Bates, 2003; Lee et al., 2019; Ross and Poirier, 2004). Our finding that  
328 translation of such polyQ proteins is sensitive to ribosome levels reveal new potential therapeutic  
329 targets. For instance, several TOR inhibitors have been generated to primarily treat cancers; the  
330 mechanism we have identified provides a potential pathway to repurpose these drugs to reduce  
331 polyQ protein aggregation in various repeat expansion disease states (Noda, 2017; Ravikumar et  
332 al., 2004; Wyttenbach et al., 2008; Yee et al., 2021).

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343  
344 **Materials and Methods**

345  
346 **Fly lines**  
347 Flies were grown at 25-29°C and dissected between 0-3 hrs and 1-3 days post-eclosion. Heat  
348 shock experiments were performed on 1 day old flies.

349  
350 The following RNAi stocks were used in this study: *nsun2 RNAi* (Bloomington #62495), *Trm7-34*  
351 *RNAi* (Bloomington #62499), *CG32281 RNAi* (Bloomington #51764), *rswl* (Bloomington #44494),  
352 *CG9386* (Bloomington #33364), *Nsun5* (Bloomington #32400), *CG11109* (Bloomington #56897),

353 *CG11447* (Bloomington #43207), *CG3021* (Bloomington #55144), *RluA-2* (VDRC #34152), *RluA-2* (VDRC #106382), *Wuho* (Bloomington #61281), *CG10903* (VDRC #57481), *RluA-1* (VDRC #41757), *RluA-1* (VDRC #41758), *RluA-1* (VDRC #109586), *Tailor* (Bloomington #36896), *Pus1* (Bloomington #53288), *NOP60B* (Bloomington #36595), *CG6745* (Bloomington #41825), *CG7637* (Bloomington #55194), *NHP2* (Bloomington #51784), *CG4038* (Bloomington #34013), *CG34140* (Bloomington #38951), *CG34140* (Bloomington #57311), *CG3645* (VDRC #107156), *CG1434* (VDRC #104876), *CG3434* (VDRC #45130), *tgt* (VDRC #41644), *AlkB* (Bloomington #43300), *Paics* (Bloomington #62241), *Ras* (Bloomington #31654), *Ras* (Bloomington #51717), *Ras* (Bloomington #31653), *pfas* (Bloomington #36686), *pfas* (Bloomington #80831), *bam RNAi*; *hs-bam* (Ohlstein, B. & McKearin, D), *RpS10b* (Bloomington #43976), *RpS19b* (VDRC #22073), *UAS-raptor-HA* (Bloomington #53726).

364

365 The following tissue-specific drivers were used in this study: *UAS-Dcr2;nosGAL4* (Bloomington #25751), *UAS-Dcr2;nosGAL4;bamGFP* (Lehmann Lab), *If/CyO;nosGAL4* (Lehmann Lab), *nosGAL4;MKRS/TM6* (Bloomington #4442), and *TjGAL4/CyO* (Lehmann Lab).

366

367 The following stocks were used in this study: *RpS5b-HA* and *UAS-Rbox1-RN* (Buszazak Lab) and *UAS-Tkv* (Bloomington#36536), *UAS-Rbox1-RN* (Buszazak Lab) *UAS-Bruno* (Ephrussi Lab) and *UAS-41Q.HA* (Bloomington #30540).

368

### 369 **Rapamycin treatment**

370 One day prior to treatment, 400  $\mu$ L of 100uM Rapamycin or 400 $\mu$ L of ethanol was added to the top of food and allowed to dry. Flies were crossed at 18°C and collected 1-2 days post-eclosion. Flies were placed on food and temperature shifted to 29°C. Every other day flies were placed onto fresh food with 400  $\mu$ L of 100uM Rapamycin or 400 $\mu$ L of ethanol for a total of 7 days. Flies were dissected as described below.

371

### 372 **Genotypes used to enrich specific stages of germline**

373 To enrich for germline Stem Cells: *nosGAL4>UAS-tkv* (Flora et al., 2018; Xie and Spradling, 2000, 1998). Cystoblasts: *nosGAL4>bam RNAi* (Chen and McKearin, 2003a, 2003b; McKearin and Spradling, 1990). Differentiating Cysts: *nosGAL4>bam RNAi; hs-bam* (McCarthy et al., 2022; Ohlstein and McKearin, 1997). Female flies were heat shocked at 37° C for 2 hours, incubated at room temperature for 4 hours and heat shocked again for 2 hours. This was subsequently repeated the next day and flies were dissected. Young Wild Type: Female flies were collected and dissected within 2 hours of eclosion. To dissect wild-type ovaries, 2-3 day old females (*UAS-dcr;nosGAL4*) were fatten overnight and dissected the next day.

374

### 375 **Dissection and Immunostaining**

376 Ovaries were dissected into 1X PBS and fixed for 10 minutes in 5% methanol-free formaldehyde (Flora et al., 2018). Samples were washed in 1 mL PBT (1X PBS, 0.5% Triton X-100, 0.3% BSA) 4 times for 10 minutes each. Primary antibodies were added in PBT and incubated at 4°C rotating overnight. Samples were washed 3 times for 10 minutes each in 1 mL PBT, and once in 1 mL PBT with 2% donkey serum (Sigma) for 10 minutes. Secondary antibodies were added in PBT with 4% donkey serum and incubated at 4°C rotating overnight. Samples were washed 4 times

397 for 10 minutes each in 1 mL of 1X PBST (0.2% Tween 20 in 1x PBS). Vectashield with DAPI  
398 (Vector Laboratories) was added for 30 minutes before mounting. The following primary  
399 antibodies were used: mouse anti-1B1 (1:20, DSHB), rabbit anti-Vasa (1:1000, Rangan Lab),  
400 chicken anti-Vasa (1:1000), rabbit anti-GFP (1:2000, Abcam, ab6556), rabbit anti-Egl (1:1000,  
401 Lehmann Lab), mouse anti-pseudouridine (1:1000, MBL Life Sciences), mouse anti-C3G (1:1000,  
402 Hawley Lab), rat Anti-HA(1:500, Roche, 11 867 423 001), mouse anti-Fibrillarin (1:50, Fuchs Lab),  
403 guinea pig anti-Rbfox1 (1:1000, Buszazak Lab) and rabbit anti-phosphorylated-S6 (1:200,  
404 Teleman Lab).The following secondary antibodies were used: Alexa 488 (Molecular Probes), Cy3,  
405 and Cy5 (Jackson Labs) were used at a dilution of 1:500.

406

#### 407 **Fluorescence Imaging**

408 The tissues were visualized under 20X oil and 40X oil objective lenses and images were acquired  
409 using a Zeiss LSM-710 confocal microscope. Confocal images were processed with ImageJ. The  
410 images were quantified using ImageJ with the Measurement function.

411

#### 412 **AU quantification of protein or in situ**

413 To quantify antibody staining intensities for Rbfox1, Bruno, GFP, and pseudouridine or in situ  
414 probe fluorescence in germ cells, images for both control and experimental germaria were taken  
415 using the same confocal settings. Z stacks were obtained for all images. Similar planes in control  
416 and experimental germaria were chosen, the area of germ cells positive for the proteins or in situ  
417 of interest was outlined and analyzed using the 'analyze' tool in Fiji (ImageJ). The mean intensity  
418 and area of the specified region was obtained. An average of all the ratios (Mean/Area), for the  
419 proteins or in situ of interest, per image was calculated for both, control and experimental.  
420 Germline intensities were normalized to somatic intensities or if the protein or in situ of interest is  
421 germline enriched and not expressed in the soma they were normalized to Vasa or background.  
422 The highest mean intensity between control and experimental(s) was used to normalize to a value  
423 of 1 A.U. on the graph.

424

425 To quantify polyQ-HA, images were first filtered with a median pixel of 1. The program set the  
426 threshold values using max entropy threshold for the images and the outline of the germline was  
427 traced using the germline marker Vasa. The percent pixel count per the germline area was found  
428 and normalized to the highest mean intensity between control and experimental(s). For rapamycin  
429 treatment, 15 control and 15 treated germaria were used. Three randomly selected slices of each  
430 stack (total of 45 slices) were quantitated for both control and rapamycin treated germaria.

431

#### 432 **Egg Laying Assay**

433 Egg laying assays were conducted in triplicate in vials containing standard fly food. Prior to the  
434 assay, dry yeast was added to each vial along with 3 adult females (all 1 day post-eclosion) and  
435 1 male . Flies were incubated at 29°C overnight. The flies were then placed in a new tube and the  
436 total number of eggs counted.

437

#### 438 **RNA Isolation**

439 Ovaries were dissected in 1X PBS and homogenized by motorized pestle in 100µL of TRIzol  
440 (Invitrogen, 15596026). RNA was isolated by adding an additional 950 µL of TRIzol and 200uL of

441 chloroform with mixing. Samples were centrifuged at 13,000 rpm, 4°C for 15 minutes. The  
442 aqueous phase was transferred to a new tube, nucleic acids were precipitated using 1 mL of  
443 100% ethanol, 52 µL of 3M sodium acetate and precipitated for >1 hour at -20°C. Samples were  
444 centrifuged at 13,000 rpm, 4°C for 20 minutes. Ethanol was decanted, pellets were washed  
445 twice with 1 mL of 70% ethanol and dried at room temperature for 10 minutes. Pellets were  
446 dissolved in 20 µL RNase free water and placed in a 42°C for 10 minutes. The concentration of  
447 nucleic acid samples were measured on a spectrophotometer. The samples were treated with  
448 DNase (TURBO DNA-free Kit, Life Technologies, AM1907) and incubated at 37°C for 30 min.  
449 DNase was inactivated using the included DNase. Inactivation reagent and buffer according to  
450 manufacturers instructions.

451

#### 452 **RNA-seq and Polysome-seq library preparation**

453 RNA was isolated as previously described above. Total RNA samples were run on a 1% agarose  
454 gel to assess sample integrity (McCarthy et al., 2018). To generate RNA-seq libraries, total RNA  
455 was incubated with poly(A) selection bead. mRNA libraries were prepared using the NEXTflex  
456 Rapid Directional RNAseq Kit (BioO Scientific Corp.). Fragmentation of the mRNA was achieved  
457 by incubating 95°C for 13 minutes to produce ~300 bp fragments. Single-end mRNA sequencing  
458 (75 base pair) was performed for each sample with an Illumina NextSeq500, carried out by the  
459 Center for Functional Genomics (CFG). The sequenced reads were aligned to the *Drosophila*  
460 *melanogaster* genome (UCSCdm6) using HISTAT2 with Refseq annotate transcripts as a guide.  
461 featureCounts was used to generate raw counts and differential gene expression was assayed  
462 by Deseq, using a false discovery rate of (FDR) of 0.05, and genes with 2-fold or greater were  
463 considered significant. Gene ontology enrichment of differential genes was performed using  
464 Panther.

465

466 Polysome profiling of ovaries was adapted from previous protocols (McCarthy et al., 2022).  
467 Approximately 100 young wild type flies (*UAS-dcr;nosGAL4*) or about 275 experimental ovary  
468 pairs Nop60B were dissected (within 2 hrs of eclosion) in 1X PBS. The ovaries were immediately  
469 flash frozen on liquid nitrogen. Samples were homogenized by motorized pestle in lysis buffer  
470 and 20% of lysate was used as input for mRNA isolation and library preparation (as described  
471 above). Samples were loaded onto 10-45% CHX supplemented sucrose gradients in 9/16 x 3.5  
472 PA tubes (Beckman Coulter, #331372) and spun at 35,000 x g in an SW41 rotor for 3 hours at  
473 4°C. Gradients were fractionated with a Density Gradient Fractionation System (#621140007).  
474 RNA was extracted using acid phenol-chloroform and precipitated overnight. Pelleted RNA was  
475 resuspended in 20 µL water, treated with TURBO DNase and libraries were prepared as  
476 described above.

477

#### 478 **Polysome-Seq Analysis**

479 Analysis of polysome-seq was done using <https://ruggleslab.shinyapps.io/RIVET/> (Ernlund et al.,  
480 2018). Polysome associated targets were further defined using the following parameters. Lowly  
481 associated mRNAs were identified by <2 fold change and <0.05 p-Value while highly associated  
482 mRNAs were identified by >2 fold change and <0.05 p-Value.

483

#### 484 **Ribosome footprinting**

485 *Ribo-Seq library preparation*

486 Ribosome footprinting was performed as previously described (Dunn et al., 2013) with several  
487 modifications. ~500  $\mu$ L of ovaries were hand-dissected in Schneider's Drosophila Medium  
488 (ThermoFisher), washed twice in 1 mL of lysis buffer (0.5% Triton X-100, 150 mM NaCl, 5 mM  
489 MgCl<sub>2</sub>, 50 mM Tris-HCl pH 7.5), and flash frozen in 2 mL of lysis buffer supplemented by 1 mM  
490 DTT, 50  $\mu$ M GMP-PNP, 2  $\mu$ g/mL emetine, and 20 U/mL Superase-In RNase Inhibitor (Ambion) in  
491 liquid N<sub>2</sub>. Ovaries were lysed using a Cellcrusher tissue pulverizer (Cellcrusher), allowed to thaw  
492 on ice, and centrifuged first at 10,000 rpm for 10 min and then at 13,200 rpm for 10 min. 300  $\mu$ L  
493 of supernatant was used for footprint library preparation, and another 300  $\mu$ L were used for  
494 poly(A)-selected mRNA-Seq library preparation. Ribosome footprints were generated by  
495 incubating the lysate with 3 U/ $\mu$ g of micrococcal nuclease (NEB) for 40 min at 25° C, then  
496 quenching by addition of EGTA to a final concentration of 6.25 mM. Ribosomes were sedimented  
497 through a 34% sucrose cushion for 2.5 hr at 33,000 rpm in a Beckman SW50 rotor, and the pellet  
498 was re-suspended in 10 mM Tris pH 7.0. RNA was extracted using TRIzol LS (Invitrogen) and  
499 size-selected (28-34 nt) on a 15% TBE-urea gel. RNA was then de-phosphorylated by incubating  
500 with T4 polynucleotide kinase (NEB) for 1 hr at 37° C, size-selected, and ligated to the 3' adapter  
501 by incubating with T4 RNA ligase 2 truncated mutant (NEB) and 1  $\mu$ g of pre-adenylated adapter  
502 (5'rAppCTGTAGGCACCATCAAT/3ddc) for 2 hr at 25° C. The ligation products were size-  
503 selected on a 10% TBE-urea gel. Reverse transcription was performed with Superscript III  
504 (Invitrogen) using the Illumina Tru-Seq RT primer:

505

506 /5Phos/AGATCGGAAGAGCGTCGTAGGGAAAGAGTGTAGATCTCGGTGGTCGC  
507 /iSp18/CACTCA/iSp18/TTCAAGACGTGTGCTCTCCGATCTATTGATGGTGCCTACAG

508

509 and the reaction was quenched by incubating with 0.1M NaOH for 20 min at 98° C. Following  
510 rRNA depletion, cDNA libraries were circularized by two sequential CircLigase (Epicentre)  
511 reactions and amplified by 9-12 PCR cycles.

512

513 *mRNA-Seq library preparation*

514 Total RNA was extracted from 300  $\mu$ L of lysate with TRIzol LS, precipitated with isopropanol,  
515 washed in ice-cold 80% ethanol, and re-suspended in 10 mM Tris-HCl pH 7.0. mRNA-Seq  
516 libraries were then prepared from poly(A)-selected mRNA according to manufacturer's  
517 instructions using the Illumina TruSeq RNA Library Prep Kit.

518

519 *Processing of sequencing data*

520 All steps were performed on the Princeton Galaxy server (galaxy.princeton.edu). Multiplexed  
521 libraries were de-multiplexed using the Barcode Splitter tool with up to 2 mismatches. Illumina  
522 Tru-Seq adapters were clipped using the Trim Galore! tool. The trimmed reads were first mapped  
523 against Drosophila rRNA sequences using Bowtie with default parameters, and the un-aligned  
524 reads were then aligned to the Drosophila genome Release 6 (dm6) using Bowtie2 with default  
525 parameters. The resulting BAM files were used for subsequent analyses.

526

527 *Peak detection*

528 The *Drosophila melanogaster* genome (dm6) was divided into 30-bp tiles and the number of reads  
529 aligned to each tile was reported using the bamCoverage tool of the deepTools 2 programming  
530 suite (Ramírez et al., 2016). Resulting bedgraph files were pre-processed to break up 30-bp tiles  
531 into 30 1-bp tiles (Script1). Peak detection was then performed in R using the Bioconductor  
532 software suite (Gentleman et al., 2004; Huber et al., 2015). Tiles were first aligned to the transcript  
533 regions by gene using the TxDb.Dmelanogaster.UCSC.dm6.ensGene annotation, rtracklayer  
534 (Lawrence et al., 2009), GenomicRanges (Lawrence et al., 2013), and BioPhysConnectoR  
535 (Hoffgaard et al., 2010) R packages (Script2). Then the distribution of coverage in the tiles aligned  
536 to each gene transcript region was fit to a normal distribution using the MASS R package  
537 (Venables et al., 2002) (Script3 and Function1). Finally, the coverage distribution and tiles aligned  
538 to each gene region were used to identify peak containing tiles (Script4 and Function2). Peak tiles  
539 from different ribosome profiling libraries were then compared (Function3) and the names,  
540 locations, and actual sequences of high confidence peaks were extracted (Script5, Script6, and  
541 Function4) using the Bsgenome.Dmelanogaster.UCSU.dm6 annotation and the Biostrings (H.  
542 Pagès, 2017) and GenomicRanges (Lawrence et al., 2013) R packages. Peaks present in at least  
543 two of the three Ribo-Seq libraries but not in the control RNA-Seq libraries at the corresponding  
544 positions were considered high confidence ribosome footprint peaks.  
545

#### 546 **Mass spectrometry**

547 Ovaries were dissected in 1X PBS and homogenized by motorized pestle in 100uL of TRIzol  
548 (Invitrogen, 15596026). RNA was isolated by adding an additional 950 uL of TRIzol and 200uL of  
549 Chloroform with mixing. Samples were centrifuged at 13,000 rpm, 4°C for 15 minutes. The  
550 aqueous phase was transferred to a new tube. Nucleic acids were precipitated by adding and  
551 equal volume of 5 M Ammonium Acetate (Sigma-Aldrich), 2.5 volumes 100% ethanol and  
552 precipitated for >1 hour at -80°C. Samples were centrifuged at 13,000 rpm, 4°C for 20 minutes.  
553 Ethanol was decanted, pellets were washed four times with 1 mL of cold 70% ethanol and dried  
554 at room temperature for 10 minutes. Pellets were dissolved in 20  $\mu$ L RNase free water and placed  
555 in a 42°C for 10 minutes.  
556

557 RNA concentration was determined by using UV 260 nm. The RNA was then treated with  
558 nuclease P1 and phosphodiesterase to obtain the desired ribonucleotide monophosphate  
559 mixtures for mass spectrometric analysis, as previously described (McIntyre et al., 2018; Rose et  
560 al., 2016, 2015).  
561

562 Immediately before analysis, the obtained mononucleotide mixtures were diluted to 4 ng/ $\mu$ L in 10  
563 mM ammonium acetate and 10% isopropanol. All samples were analyzed on a Thermo Scientific  
564 LTQ-Orbitrap Velos instrument as previously described (Rose et al., 2016, 2015; McIntyre et al.,  
565 2018). Analyses were accomplished by using direct infusion electrospray ionization (ESI) in  
566 negative ion mode.  
567

568 The relative abundance of each RNA PTM was expressed as Abundance versus Proxy (AvP),  
569 which was calculated according to the following equation:  $AvP_x = \frac{a_{ix}}{\sum_1^4 cr_i} \times 100$  in which the signal  
570 intensity ( $a_{ix}$ ) of each RNA PTM was normalized against the sum of the intensities displayed in  
571 the same spectrum by the four canonical bases ( $cr_i$ ).  
572

572  
573 The RNA PTM profiling table translates relative abundances in AvP units to a hot-cold color  
574 gradient. The relative abundances displayed by the samples in the first column on the left were  
575 used as the baseline for comparisons with the rest of the samples. A different color was assigned  
576 only if the respective values were statistically different according to an unpaired student *t*-test with  
577 a *P*-value < 0.05. Each data point was the result of three to five biological replicates, which were  
578 each separately analyzed three times (technical replicates). Therefore, each value represents the  
579 average and standard deviation of a total of 9 to 15 separate analyses.  
580

581 Tandem mass spectrometry was carried out in negative mode to differentiate uridine and  
582 pseudouridine (Rose et al., 2016, 2015; McIntyre et al., 2018). The contribution of each isomer to  
583 the initial signal can be estimated from the relative intensities of their unique fragments. The  
584 abbreviations and complete names of each PTM in this study are available from MODOMICS  
585 (<http://genesilico.pl/modomics/>) database.  
586

### 587 **Germline ribosome pulldowns**

588 Ribosomal pulldowns were performed as previously described with the following modifications  
589 (Chen and Dickman, 2017). Approximately 50 young wild type ovaries (UAS-dcr;nosGAL4) and  
590 ~100 Nop10<sup>55194</sup> RNAi ovaries were dissected in PBS. After lysis in ribosomal lysis buffer, 120 uL  
591 was collected for input and Trizol extraction with previously described for mass spectrometry. The  
592 remaining lysate was divided into 180 uL aliquots. 6 ug of rabbit IgG (Jackson Immunoresearch)  
593 or rat-HA antibodies for 3 hours with rotation at 4°C. At hour 2, 50 uL Dynabeads A (Thermofisher)  
594 per replicate. The beads were prepped by performing four washes using a magnetic rack (500 uL  
595 for 2 minutes each) with ribosomal lysis buffer. After the fourth wash the beads were resuspended  
596 in 50 uL of ribosomal lysis buffer. To the samples either 25 uL of IgG or anti-HA was added and  
597 left overnight with rotation at 4°C. The following day, the beads were washed with 200 uL of  
598 ribosome lysis buffer for a total of four washes. After the final wash the beads were resuspended  
599 in 15uL of ribosome lysis buffer. A Trizol extraction was performed as previously described for  
600 mass spectrometry. After RNA extraction, a small portion of the RNA was run on a 1% agarose  
601 gel to confirm the presence of rRNA.  
602

603 After the overnight incubation, the beads were washed with 200 uL of ribosome lysis buffer for a  
604 total of four washes. After the final wash the beads were resuspended in 15uL of ribosome lysis  
605 buffer. To the sample 4X SDS buffer was added and then heated at 95°C for 5 minutes and stored  
606 at -20°C until Western analysis.  
607

### 608 **Western Blot**

609 Ovaries were dissected in 1X PBS (Flora et al., 2018). After dissection, PBS was aspirated and  
610 30 uL of RIPA buffer with protease inhibitors was added, and the tissue was homogenized. The  
611 homogenate was centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous layer was  
612 transferred into a new tube while avoiding the top lipid layer. 1 uL of the protein extract was used  
613 to determine protein concentration via Invitrogen Qubit® Protein Assay Kit. 15-20 µg of protein  
614 was denatured with 4X Laemmli Sample Buffer and β-mercaptoethanol at 95°C for 5 minutes. The  
615 samples were loaded onto a Mini-PROTEAN TGX 4-20% gradient SDS-PAGE gels and run at

616 300V for 20 minutes. The proteins were then transferred to a 0.20  $\mu$ m nitrocellulose membrane  
617 using Bio-Rad Trans-blot Turbo System. After transfer, the membrane was blocked in 5% milk in  
618 PBST for 2 hours at RT. The following antibodies were used: rat-HA (1:4000), rabbit-Vasa  
619 (1:6000) and rabbit-RpL26 (1:1000). Primary antibody was prepared in 5% milk in PBST was  
620 added to the membrane and incubated at 4°C overnight. The membrane was then washed three  
621 times in 0.5% milk PBST. Anti-rabbit HRP (1:10,000) or Anti-rabbit HRP (1:10,000) was prepared  
622 in 5% milk in PBST, and was added to the membrane and incubated at room temperature for 1  
623 hour. The membrane was then washed 3 times in PBST. The Bio-Rad chemiluminescence ECL  
624 kit was used to image the membrane.

625

626 Note: To help normalize germline in the Western Blot probing for the PolyQ-HA reporter, first 15-  
627 20 ug of lysate run and probed for Vasa. Controls were then diluted 1:5 to help equalize the  
628 amount of germline loaded and compared to the H/ACA box member knockdown. Normalizations  
629 were performed using the top Vasa band.

630

### 631 **Stellaris *in situ* hybridizations**

632 A modified *in situ* hybridization procedure for *Drosophila* ovaries was followed from (Sarkar et al.,  
633 2021). Probes were designed and generated by LGC Biosearch Technologies using Stellaris®  
634 RNA FISH Probe Designer, with specificity to target base pairs of target mRNAs. Ovaries (3 pairs  
635 per sample) were dissected in RNase free PBS and fixed as described above. The fixed tissue  
636 was washed with twice with 1 mL of PBS and then permabilized with 70% ethanol at 4°C for 2  
637 hours. After permeabilization, 1 mL of wash buffer was added (40 mL RNase free water, 5 mL  
638 deionized formamide and 5 ML 20X SSC) for a 5 minute wash. To the sample 50  $\mu$ L of a Stellaris  
639 Hybridization buffer, 10% (vol.vol.) of formamide with 50-100 nm of oligos and properly diluted  
640 antibodies were added and incubated at 30°C for a minimum of 16 hours in the dark. After the  
641 overnight incubation, the sample was washed twice with 1 mL of wash buffer with properly diluted  
642 secondary antibodies for 30 minutes. After the second wash Vectashield was added and samples  
643 were imaged.

644

645 Stellaris probes were designed on (<https://www.biosearchtech.com/support/education/stellaris-rna-fish>) to all possible isoforms and Cy3 probe. Sequences found in excel file  
646 stellarisinsitu probes.xlsx.  
647

648

### 649 **Quantitative Real Time-PCR (qRT-PCR)**

650 Once RNA was purified and isolated, see RNA Isolation section, a reverse transcription (RT) was  
651 performed using Superscript II according to the manufacturer's protocol with equivalent volumes  
652 of RNA for each sample. cDNA was amplified using 5  $\mu$ L of SYBR green Master Mix, 0.3  $\mu$ L of  
653 10  $\mu$ M of each reverse and forward primers in a 10  $\mu$ L reaction. For each sample 3 biological and  
654 a minimum of 2 technical replicates were performed. Technical replicates were averaged, and  
655 tubulin was utilized as a control. To calculate fold change relative to tubulin mRNA levels, the  
656 average of the 2 $^{-\Delta Ct}$  for the biological replicates was calculated with error bars representing  
657 Standard Error of the Mean. Statistics were performed using a paired t-test on  $\Delta Ct$  values.  
658

659

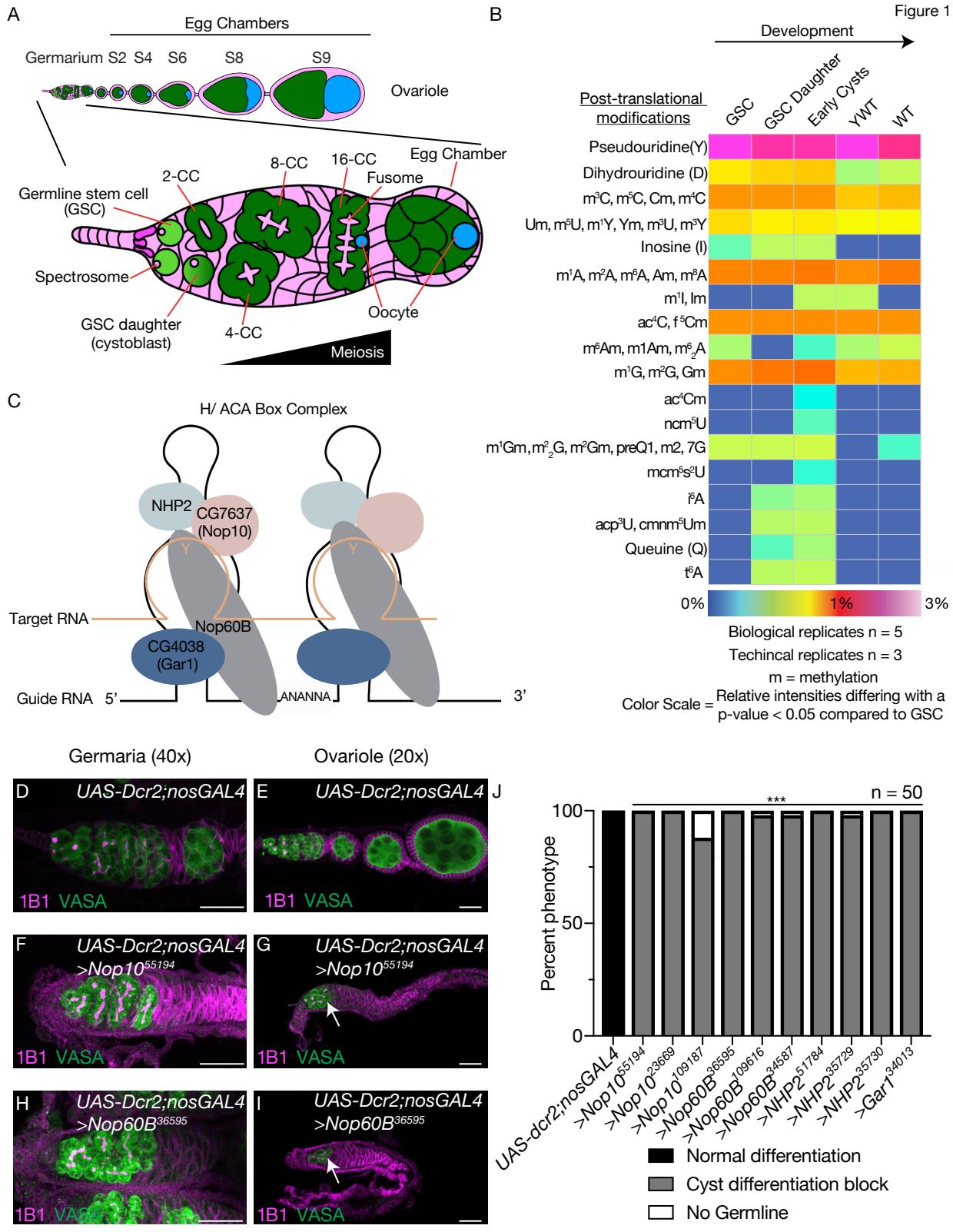
### **MEME Analyses**

660 The 5'UTR, CDS, 3'UTR and amino acid sequence of 465 mRNAs that are lowly associated with  
661 polysome than control and 320 mRNAs highly associated with polysome and analyzed by the  
662 MEME algorithm (Bailey, n.d.). Discriminative mode analysis was conducted against 1573 non-  
663 target gene sequences as background with default parameters. Motif logos, number of sites, and  
664 E-values all reported as produced by output of the program.

665

### 666 **FIMO Analyses**

667 An amino acid motif of 5Qs was run against the amino acid sequences of all mRNAs that were  
668 lowly associated with polysome. Motifs identified in targets were searched in the given strand with  
669 a p-value < 1E-4.

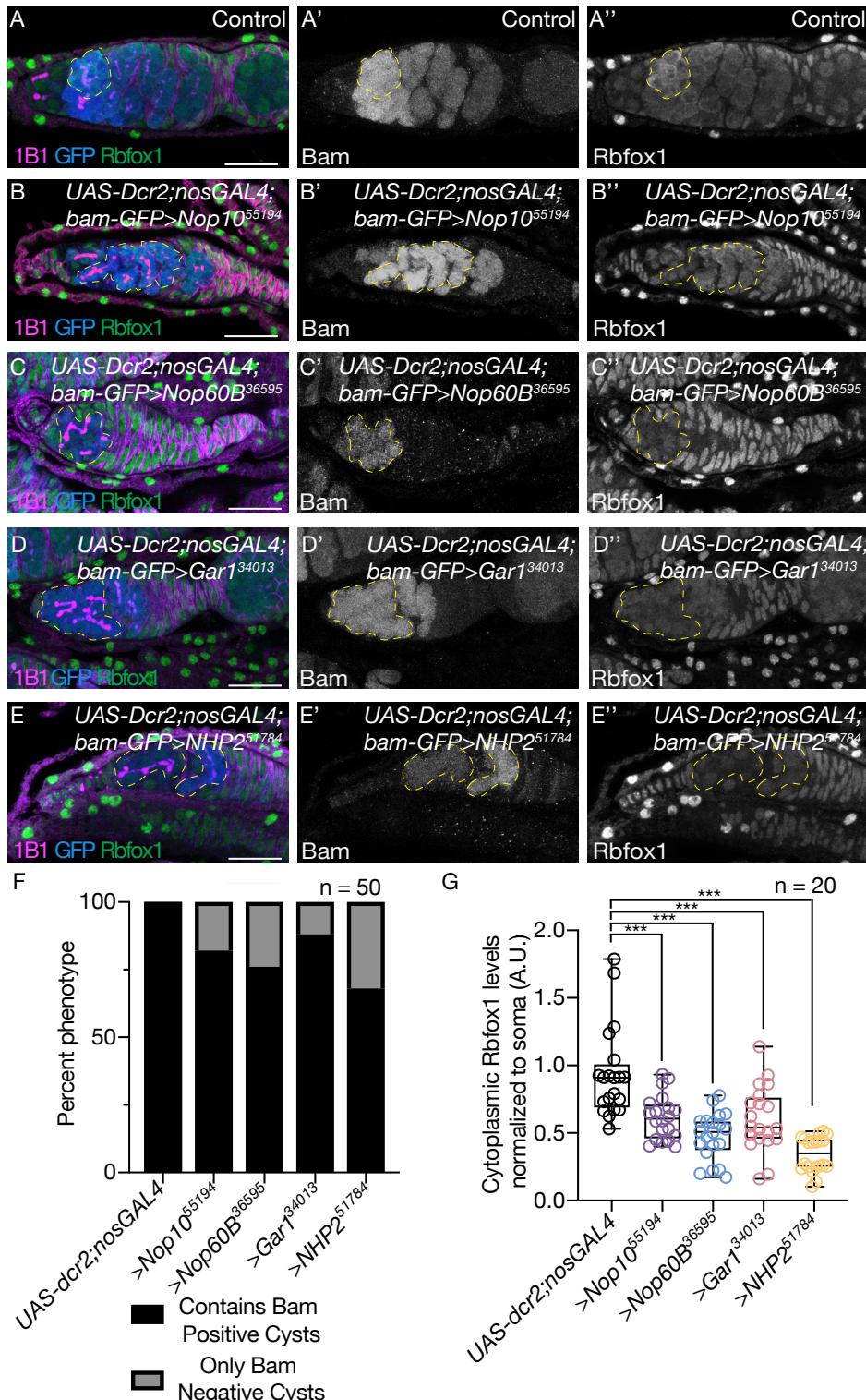


670  
671

**Figure 1: Pseudouridine is a critical modification required for oogenesis**

672 (A) Schematic of a Drosophila ovariole and germarium. The germarium is present at the anterior  
673 tip of the ovariole and goes through successive stages of egg chamber development. The  
674 germline stem cells (GSC) (green) reside at the anterior tip of the germarium and are surrounded  
675 by somatic cells (magenta). The GSC divides to give rise to a GSC daughter or cystoblast (CB).  
676 The CB on the differentiation factor and undergoes incomplete mitotic divisions to give rise to a  
677 2-, 4-, 8-, and 16- cell cyst (differentiating cysts). During the cyst stages, the germline transition  
678 from a mitotic fate to meiotic fate. The single cells are marked by spectrosomes (magenta) and  
679 the cysts are marked by the branched structure called fusomes (magenta). The 16- cell cyst buds  
680 off from the germarium and is encapsulated by the soma to generate an egg chamber. One of the  
681 16- cells is designated as the oocyte (blue), going through successive egg chamber  
682 developmental eventually forming a mature egg.  
683 (B) Heat map of mass spectrometry analysis of RNA modifications obtained from total RNA  
684 extracts from germaria enriched for GSCs, GSC daughters, cysts, YWT, and Wild Type (WT). A  
685 heat map covers relative abundances up to 3%. The different colors express variations of relative  
686 abundance compared to the GSC with a  $p < 0.05$  statistical significance. For each developmental  
687 stage 5 biological replicates were analyzed with 3 technical replicates of each biological  
688 replicates.  
689 (C) The H/ACA box is composed of four proteins CG4038 or Gar1 (dark blue), Nop60B (gray),  
690 NHP2 (light blue) and CG7637 or Nop10 (salmon). The H/ACA box uses a small RNA guide that  
691 corresponds to the target RNA where it complex deposits pseudouridine.  
692 (D, E) Images of 40x *UAS-Dcr2;nosGAL4* (driver control) germarium (D) and 20x *UAS-*  
693 *dcr2;nosGAL4* (driver control) ovarioles (E) stained with anti-1B1 (magenta) and anti-Vasa  
694 (green).  
695 (F, G) Images at 40x (F) and 20x (G) of germarium where *Nop10* is depleted in the germline and  
696 stained with anti-1B1 (magenta) and anti-Vasa (green) resulting in a cyst progression defect.  
697 White arrow marks cyst defect in germline depleted of H/ACA box members. Scale bar for all  
698 images is 20  $\mu$ m.  
699 (H, I) Images at 40x (H) and 20x (I) of germarium where *Nop60B* is depleted in the germline and  
700 stained with anti-1B1 (magenta) and anti-Vasa (green) resulting in a cyst progression defect.  
701 White arrow marks cyst defect in germline depleted of H/ACA box members. Scale bar for all  
702 images is 20  $\mu$ m.  
703 (J) Quantification of oogenesis defect phenotypes per genotype of H/ACA box germline depletion.  
704 Statistical analysis performed with Fisher's exact test ( $n = 50$  for all, \*\*\*  $p < 0.0001$ ).  
705

Figure 2



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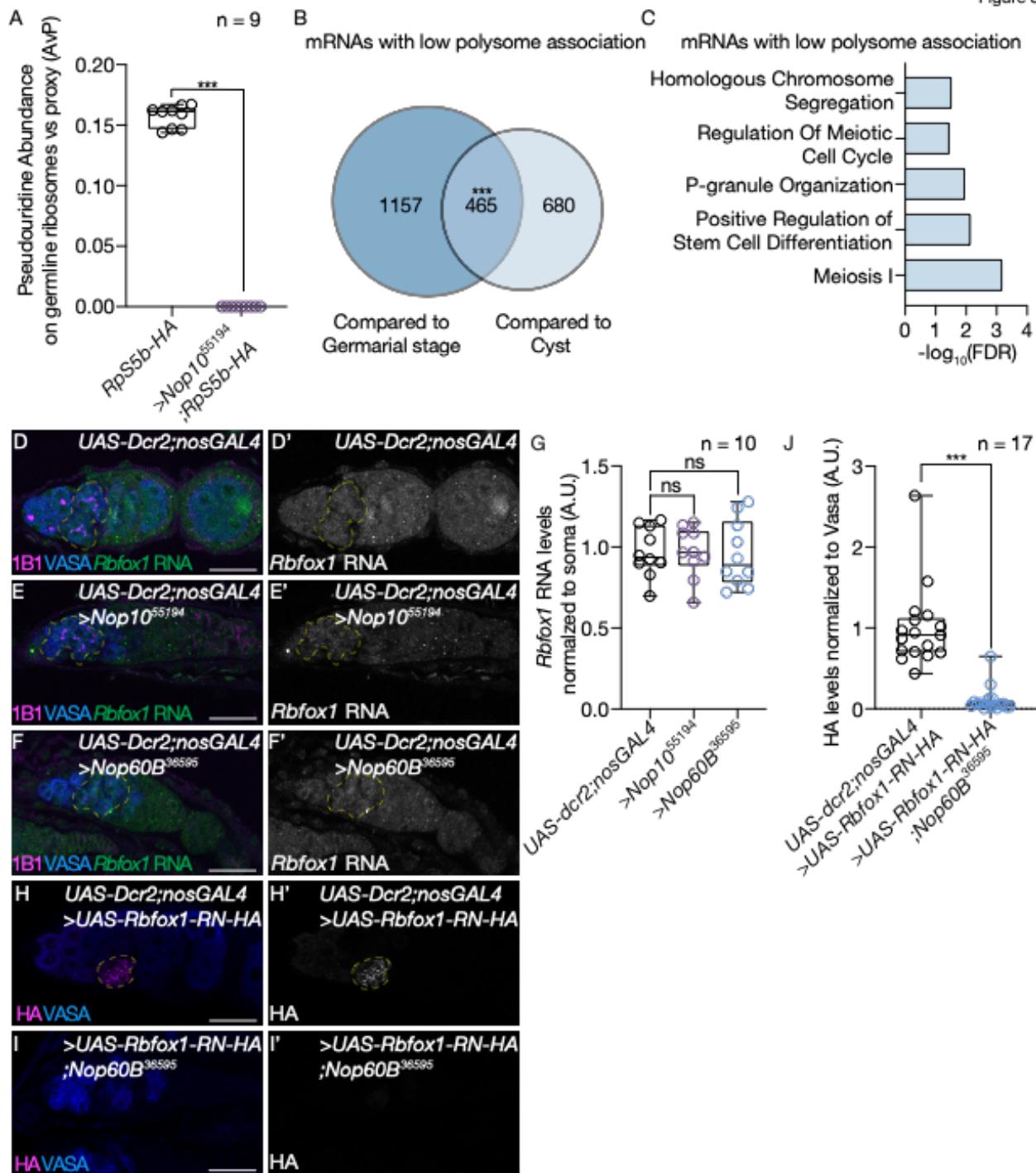
710

**Figure 2: The H/ACA box is required for proper cyst differentiation and meiotic progression**

(A-E'') *UAS-Dcr2;nosGAL4;bam-GFP* (driver control) germaria (A) and germline depletion of *Nop10* (B), *Nop60B* (C), *Gar1* (D), and *NHP2* (E) stained with anti-1B1 (magenta), anti-GFP (blue) and anti-Rbfox1 (green). GFP (A', B', C', D', E') and Rbfox1 (A'', B'', C'', D'', E'') are shown in gray

711 scale. Yellow dotted lines outline cysts that are positive for GFP but have lower Rbfox1 levels for  
712 all images. Scale bar for all images is 20  $\mu$ m.  
713 (F) Quantification of oogenesis defect phenotypes per genotype. Loss of the H/ACA box results  
714 in Bam positive cysts. Statistical analysis performed with Fisher's exact test (n = 50 each, \*\*\*  
715 p<0.0001).  
716 (G) Quantification of cytoplasmic Rbfox1 levels normalized to soma in germline depleted of  
717 *Nop10*, *Nop60B*, *Gar1* or *NHP2*. Loss of H/ACA box results in lower levels of Rbfox1 levels.  
718 Statistics performed were Dunnett's multiple comparisons test post-hoc test after one-way  
719 ANOVA (n = 20 each, \*\*\* p<0.0001).  
720

Figure 3



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**Figure 3: The H/ACA box is required for translation of meiotic mRNAs**

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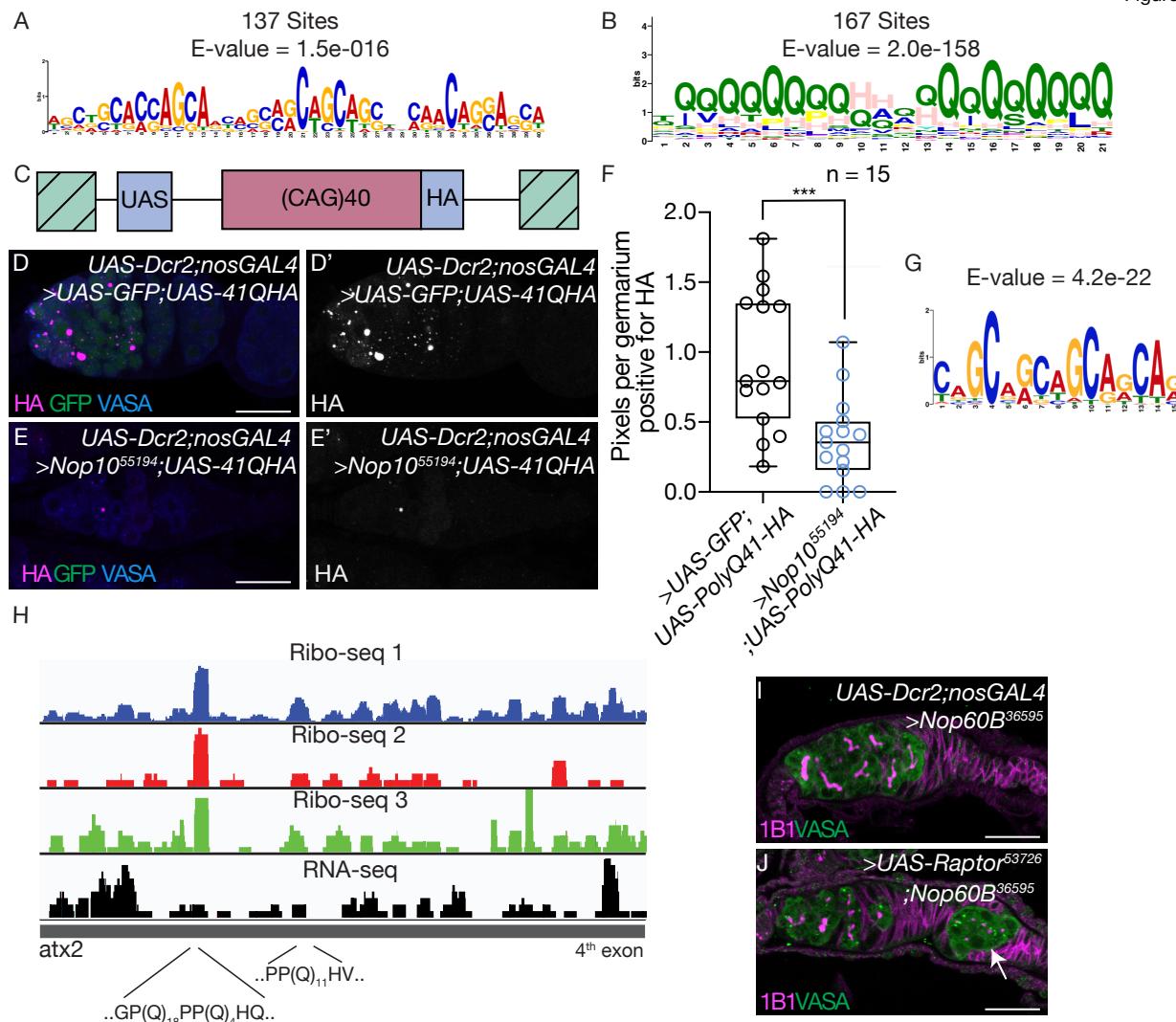
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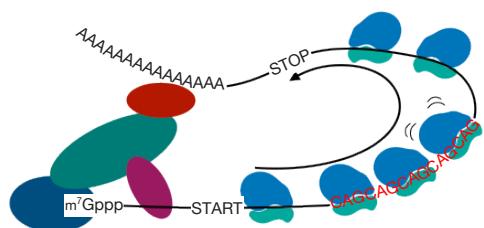
(A) Mass spectrometry analysis of rRNA isolated from germline ribosomal pulldowns showing reduced pseudouridine levels on rRNA. Statistics performed were t-test of pseudouridine levels comparing germaria enriched for cysts and *Nop10* depleted germaria. For each developmental stage at least 2 biological replicates were analyzed with 3 technical replicates of each biological replicates (\*\* p<0.0001).

728 (B) Venn diagram illustrating overlap of Nop60B-polysome <-2 fold less association with the  
729 ribosome upon loss of *Nop60B* ( $n = 2$ ,  $e < 2.87 \times 10^{-192}$ , Hypergeometric Test). Controls were  
730 cysts, enriched through heat shock, and young wild-type (YWT), which includes germarial stages  
731 and a few egg chambers.  
732 (C) Significant biological process GO terms of shared lowly associated mRNAs in  
733 ovaries depleted of *Nop60B* compared to control sets, showing an enrichment for mRNAs  
734 associated with meiosis 1.  
735 (D) In situ hybridization to *Rbfox1* RNA (green) and staining with anti-1B1 (magenta) and anti-  
736 Vasa (blue) in *UAS-Dcr2;nosGAL4* (driver control) germlaria and (E,F) germline-depleted of  
737 *Nop10* and *Nop60B*. *Rbfox1* RNA is shown in gray scale (D', E', F'). Yellow dotted line outlines  
738 *Rbfox1* RNA.  
739 (G) Quantification of *Rbfox1* RNA levels in germline depleted of varying members of *Nop10* and  
740 *Nop60B* normalized to soma showing no significant difference in *Rbfox1* levels. Statistics  
741 performed were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA ( $n = 10$   
742 for all, not significant,  $P > 0.9999$  and  $p = 0.9792$  respectively).  
743 (H) Germlaria of *UAS-Dcr2;nosGAL4* (driver control) driving UAS-*Rbfox1*-RN-HA and (I) germline  
744 depleted of *Nop60B* driving *Rbfox1*-RN-HA. Germlaria stained with anti-Vasa (blue) and anti-HA  
745 (magenta). HA is shown in gray scale (H', I').  
746 (J) Quantification of HA levels in control vs germline depleted of *Nop60B* normalized to vasa  
747 showing reduced *Rbfox1*-RN-HA levels in the germline. Statistics performed were unpaired t-test  
748 ( $n = 17$  for all, \*\*\*  $p < 0.0001$ ). Yellow dotted line outlines *Rbfox1*-RN-HA.  
749

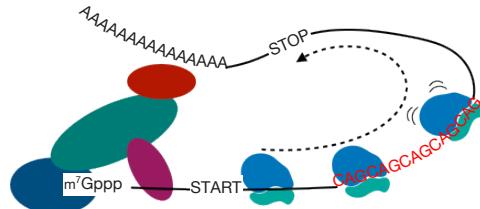
Figure 4



**K** Sufficient ribosome levels



Insufficient ribosome levels

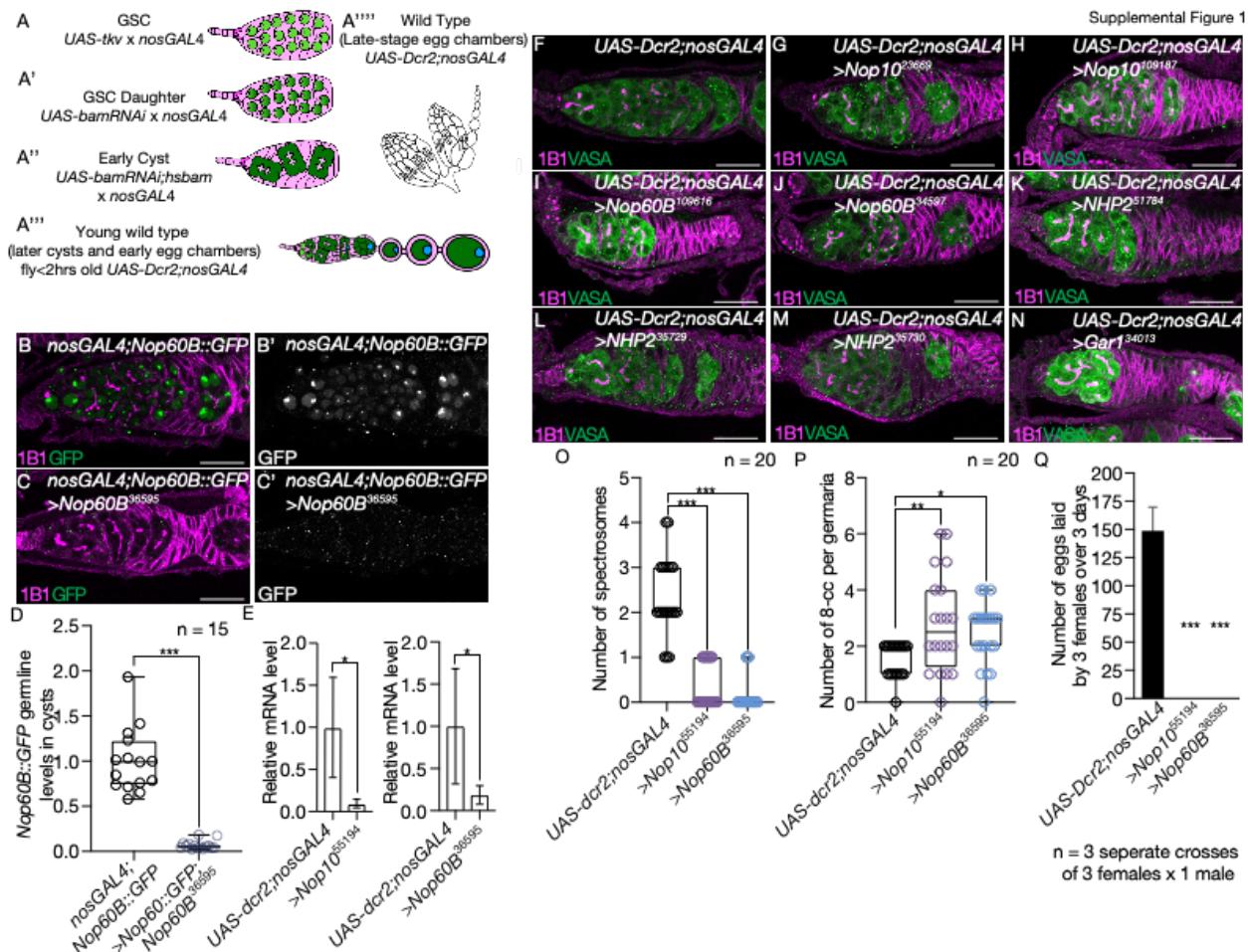


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### Figure 4: The H/ACA box is required for translating polyQ proteins

(A) Repeating CAG motif identified by MEME enriched in the CDS of 137 mRNAs that are lowly associated with polysomes in germaria depleted of *Nop60B*.  
 (B) Repeating polyQ motif present in 167 sites identified by MEME enriched that are present in the mRNAs that are lowly associated with the ribosome in germaria depleted of *Nop60B*.

756 (C) Schematic of CAG reporter which codes for a 41Q protein with an HA tag and which can be  
757 driven by the *UAS-GAL4* in a tissue specific manner.  
758 (D) Confocal image of poly41Q-HA reporter driven in *UAS-Dcr2;nosGAL4* controls flies or  
759 poly41Q-HA reporter driven in *Nop10* depleted germlaria (E) stained with anti-HA (magenta), anti-  
760 GFP (green) and anti-Vasa (blue). UAS-GFP was also driven to ensure equal GAL4 dosage. HA  
761 is shown in gray scale (D' and E'). Scale bar for all images is 20  $\mu$ m.  
762 (F) Quantification of percent of pixels per area of HA in control vs germline depleted of *Nop10*  
763 showing a reduction of HA signal in *Nop10* depleted germlaria. Statistics performed were unpaired  
764 t-test (n = 15 for all, \*\*\* p=0.0007).  
765 (G) Repeating CAG motif identified by MEME at peak regions in mRNAs detected by ribosome  
766 footprinting.  
767 (H) Ribosome footprint distribution on *atx2* mRNA, illustrating a peak in exon 4 found in common  
768 among the 3 Ribo-Seq libraries (blue, red and green) but not in the RNA-Seq library (black). A  
769 polyQ stretch is present at the ribosome peak.  
770 (I, J) Germlaria depleted for *Nop60B* (I) or *Nop60B* (J) while simultaneously overexpressing  
771 Raptor-HA using *UAS-Dcr2;nosGAL4*. Germlaria stained with anti-Vasa (green) and anti-1B1  
772 (magenta). Arrow points at egg chamber. (>*Nop60B* RNAi, N = 91, 1.1% contained 1<sup>st</sup> egg  
773 chamber while >*Nop60B* RNAi;UAS-Raptor, N = 151, 9.9% contained 1<sup>st</sup> egg chamber, Fisher's  
774 exact test, p=0.0037).  
775 (K) Representative model showing that a sufficient level of ribosomes is required for translation  
776 of meiotic mRNAs containing a repeating CAG motif. Proper ribosome levels allow for translation  
777 of these mRNAs to promote terminal differentiation. Ribosome insufficiency result in reduced  
778 translation of meiotic mRNAs, due to ribosome stalling or slowing, that contain the repeating CAG  
779 motif causing a failure of terminally differentiate.  
780



781

782 **Supplemental 1: The H/ACA box is required in the germline for proper oogenesis**

783 (A-A'') Schematic of method to enrich for GSCs (A), GSC daughters (A'), cysts (early cysts; A''),  
784 young wild type (later cysts and early egg chambers; A''), and late-stage egg chambers (A''').  
785 (B,C) nosGAL4;Nop60B::GFP (driver control) germarium (B) and germarium with germline  
786 knockdown of Nop60B in Nop60B::GFP (C) stained with anti-1B1 (magenta) and anti-GFP  
787 (green). GFP is shown in gray scale (B' and C'). Scale bar for all images is 20  $\mu$ m.

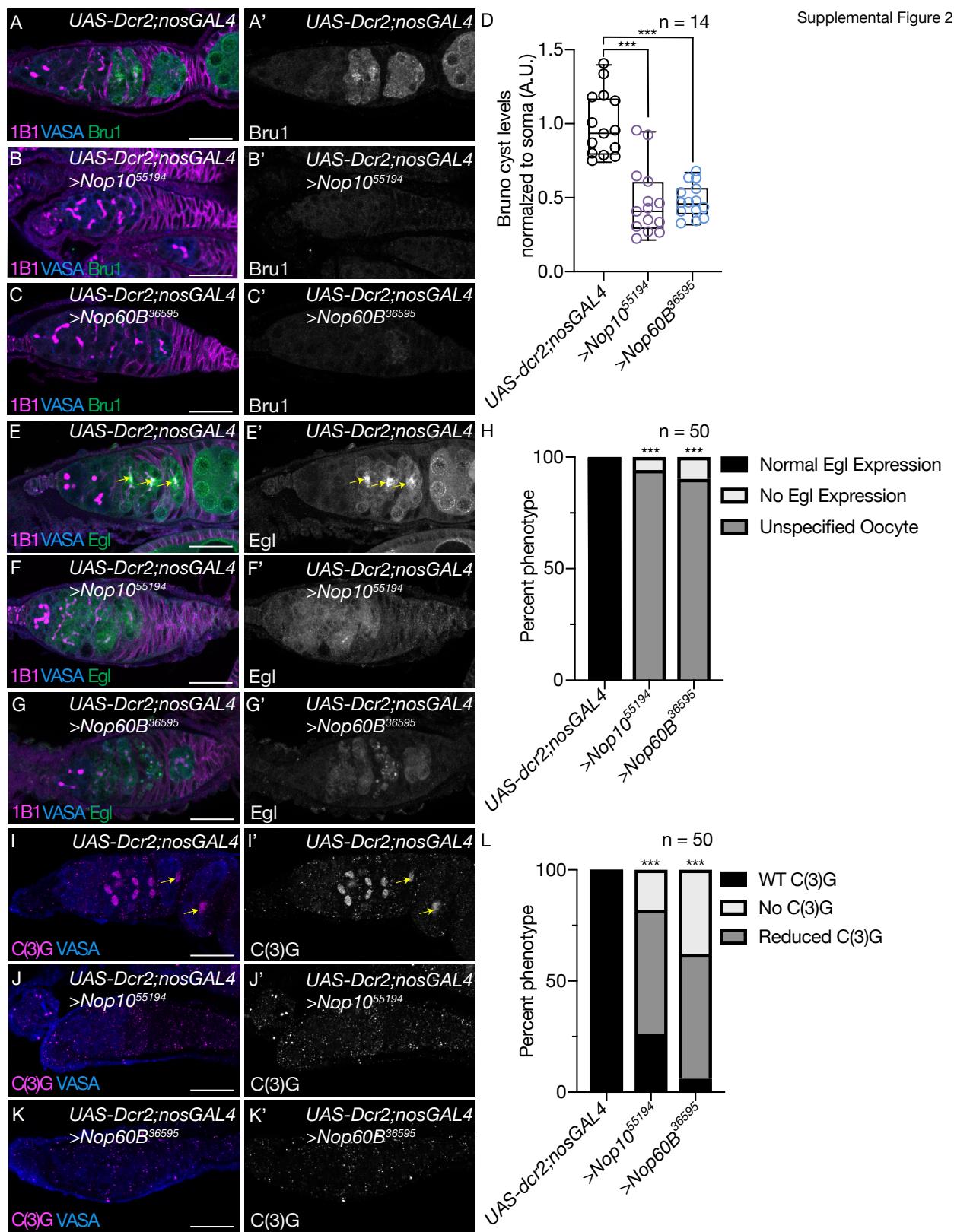
788 (D) Quantification using unpaired t-test of GFP in cysts of nosGAL4;Nop60B::GFP and germline  
789 knockdown of Nop60B in Nop60B::GFP background (n = 15 each, \*\*\* p<0.0001). There were  
790 lower levels of germline GFP in Nop60B knockdown germaria.

791 (E) qRT-PCR assaying the RNA levels of Nop10 or Nop60B in germline RNAi normalized to  
792 control, UAS-Dcr;nosGAL4, and indicating successful knockdown of H/ACA box members (n = 3,  
793 Nop10: \* p = 0.0231, paired t-test) (n=3, Nop60: \* p = 0.0142, paired t-test). Error bars  
794 representing SEM.

795 (F-N) UAS-Dcr2;nosGAL4 (driver control) germaria (F) and germline depletion of varying  
796 members of the H/ACA box stained with anti-1B1 (magenta) and anti-Vasa (green): Nop10 (G-H);  
797 Nop60B (I-J); NHP2 (K-M); and Gar1 (N). Germline knockdown of H/ACA box members  
798 results in a block in cyst development. Scale bar for all images is 20  $\mu$ m.

799 (O) Quantification of number of spectrosomes in UAS-Dcr2;nosGAL4 (driver control) germaria  
800 and germline depleted of Nop10 or Nop60B showing a loss of GSCs. Statistics performed were

801 Dunnett's multiple comparisons test post-hoc test after one-way ANOVA (n = 20 each, \*\*\*  
802 p<0.0001).  
803 (P) Quantification of number of 8-cell cysts in *UAS-Dcr2;nosGAL4* (driver control) germline and  
804 germline depleted of *Nop10* or *Nop60B* showing an increase in 8-cell cysts. Statistics performed  
805 were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA (n = 20 each,  
806 \*p=0.0341, \*\*p=0.0030,).  
807 (Q) Egg laying assay after germline RNAi knockdown of *Nop10* or *Nop60B* indicating a loss of  
808 fertility compared to *UAS-dcr2;nosGAL4* (driver control) (n = 0-173, \*\*\* p<0.001) Dunnett's  
809 multiple comparisons test post-hoc test after one-way ANOVA, p < 0.0001. Error bars are  
810 standard deviation (SD).  
811

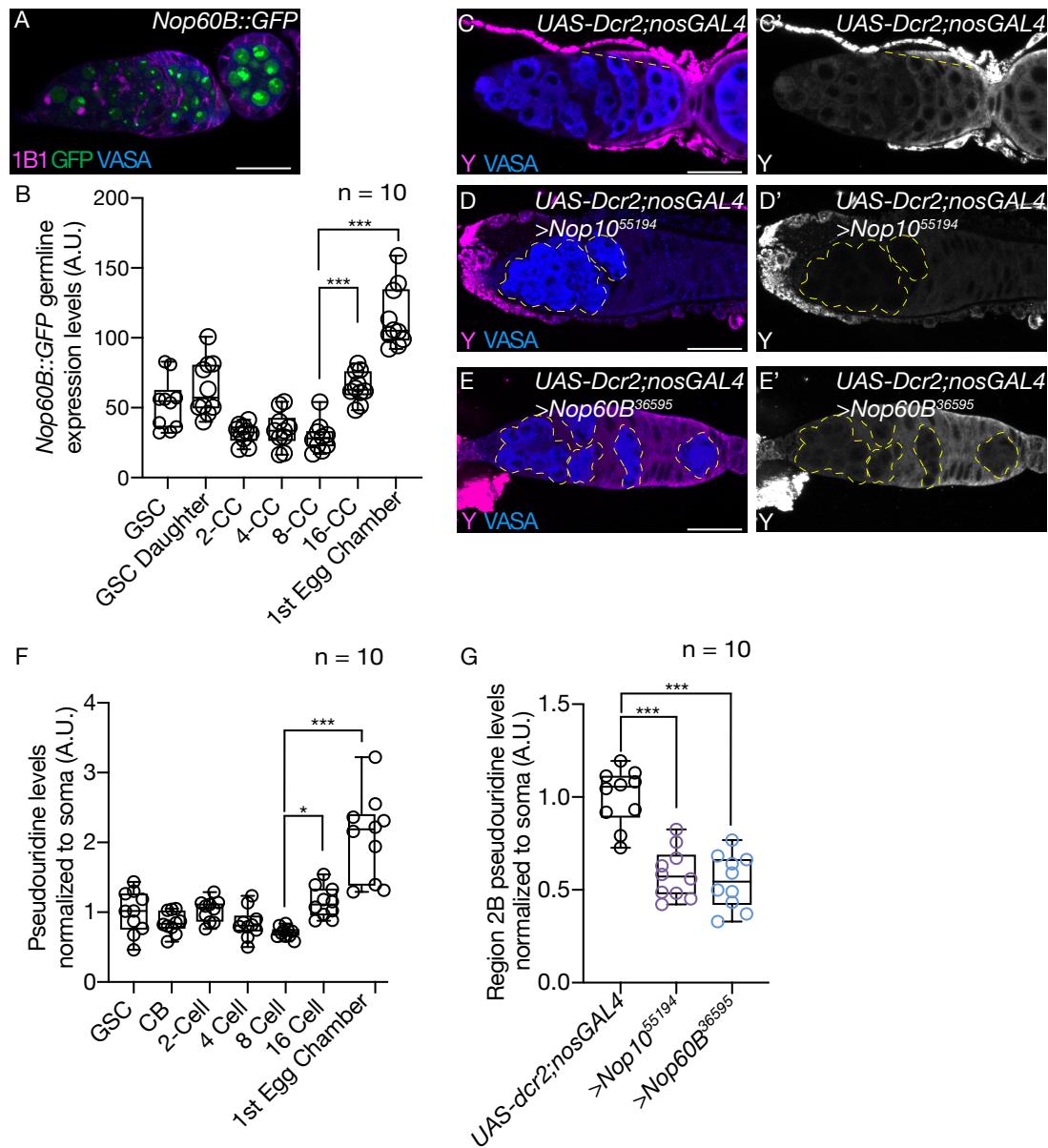


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**Supplemental 2: The H/ACA box is required for meiotic progression**

814 (A-C) *UAS-Dcr2;nosGAL4* control germaria (A) and germline depleted for *Nop10* and *Nop60B* (B,  
815 C) stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-Bru1 (green). Bru1 is shown in gray  
816 scale (A', B', C').  
817 (B) Quantification of Bru1 levels in germline depleted of *Nop10* and *Nop60B* normalized to soma  
818 showing a reduction in Bru1 levels in germaria depleted of the H/ACA box. Statistics performed  
819 were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA (n = 50 each, \*\*\*  
820 p<0.0001).  
821 (E) *UAS-Dcr2;nosGAL4* (driver control) germaria and (F, G) germline depleted of *Nop10* and  
822 *Nop60B*, stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-Egl (green). Egl is shown in  
823 gray scale (E', F', G'). Arrow pointing at designated oocyte. Scale bar for all images is 20  $\mu$ m.  
824 (H) Quantification of oogenesis defect phenotypes showing a loss of oocyte specification in  
825 germaria depleted of the H/ACA box. Statistical analysis performed with Fisher's exact test (n =  
826 50 each, \*\*\* p<0.0001).  
827 (I-K) *UAS-Dcr2;nosGAL4* (driver control) germaria (I) and germline depleted of *Nop10* (J) or  
828 *Nop60B* (K) stained with anti-c(3)G (magenta) and anti-Vasa (blue). c(3)G is shown in gray scale  
829 (I', J', K'). Arrow points to designated oocyte. Scale bar for all images is 20  $\mu$ m.  
830 (L) Quantification of oogenesis defect phenotypes showing a loss of c(3)G expression in germaria  
831 depleted of the H/ACA box. Statistical analysis performed with Fisher's exact test (n = 50 each,  
832 \*\*\* p<0.0001).  
833

Supplemental Figure 3



834

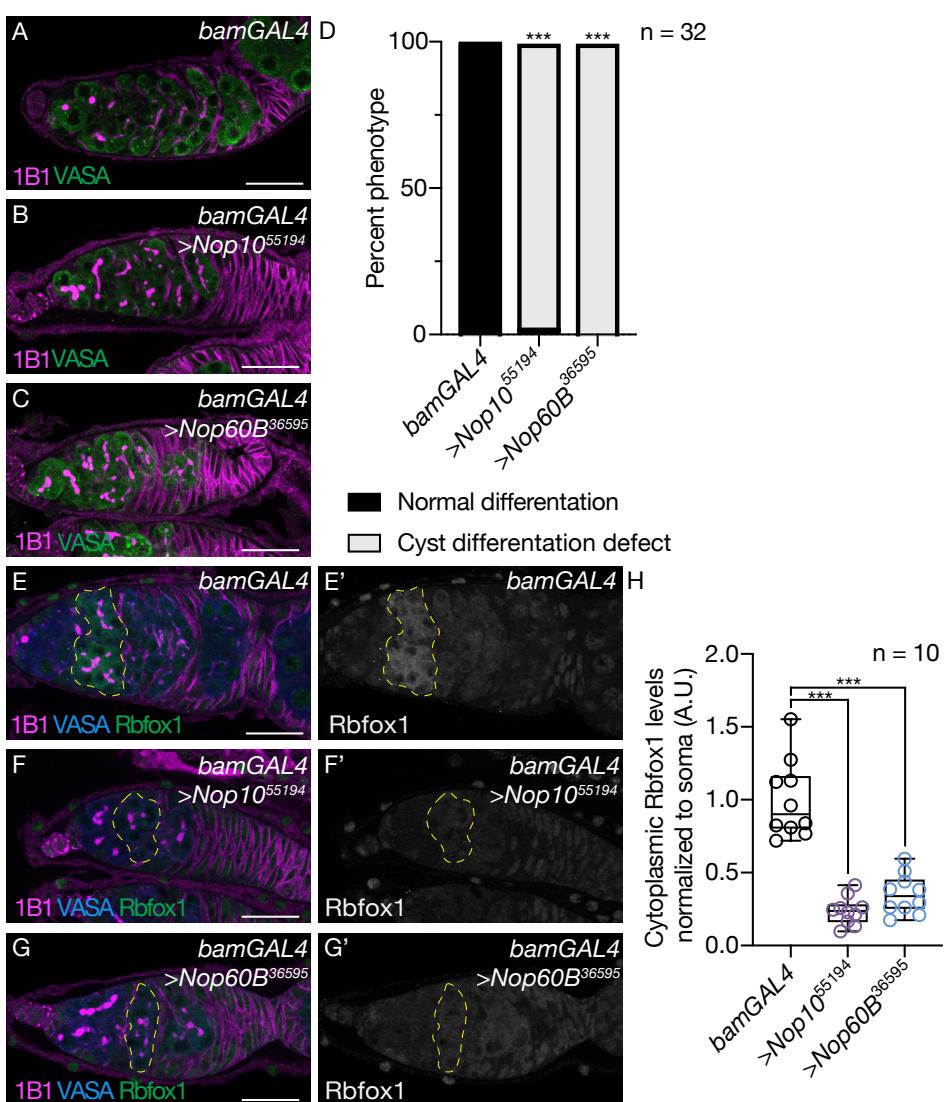
835 **Supplemental 3: *Nop60B* and pseudouridine increase during transition from cyst to egg**  
836 **chamber**

837 (A) *Nop60B::GFP* gerarium stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-GFP  
838 (green). GFP is shown in gray scale (A'). Image is taken at 40x and scale bar for all images is 20  
839  $\mu\text{m}$ .

840 (B) Quantitation of GFP from GSC to 1<sup>st</sup> egg chamber in *Nop60B::GFP*. Statistics performed were  
841 Tukey's multiple comparisons test post-hoc test after one-way ANOVA. Statistics shown  
842 comparing 8-cell cyst to 16-cell cyst and to the egg chamber showing an increase in GFP levels  
843 ( $n = 10$ , \*\*\*  $p < 0.001$ ).

844 (C) *UAS-Dcr2;nosGAL4* (driver control) gerarium and (D, E) germline depletion of *Nop10* and  
845 *Nop60B* stained with anti-pseudouridine (magenta) and anti-Vasa (blue). Pseudouridine is shown

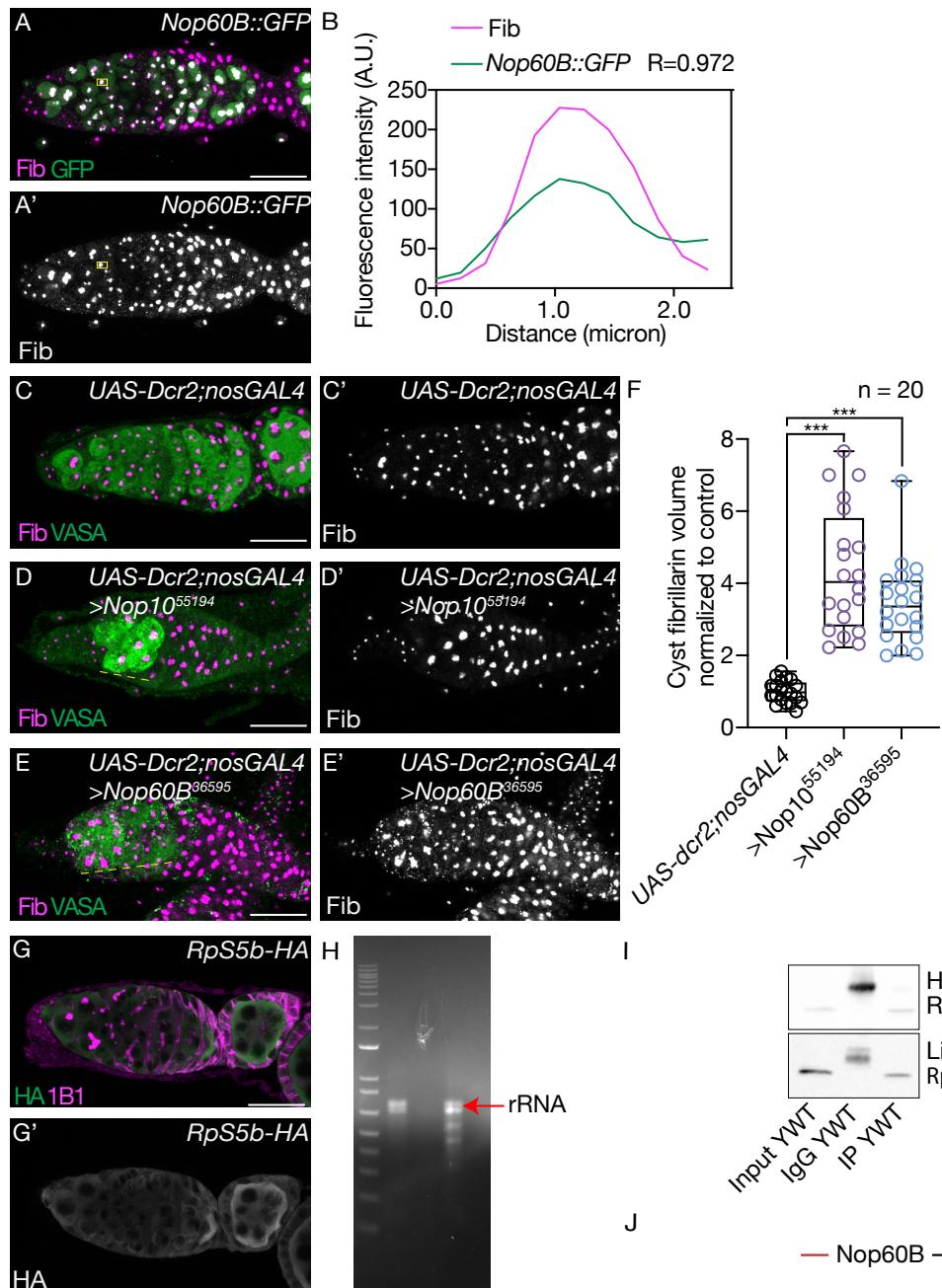
846 in gray scale (C', D' and E'). Yellow dotted line in control represents area of increasing  
847 pseudouridine levels while yellow outline in *Nop10* and *Nop60B* represents loss of pseudouridine.  
848 Scale bar for all images is 20  $\mu$ m.  
849 (F) Quantification of pseudouridine levels from GSC to 1<sup>st</sup> egg chamber in *UAS-Dcr2;nosGAL4*.  
850 Statistics performed were Tukey's multiple comparisons test post-hoc test after one-way ANOVA.  
851 Statistics shown comparing 8-cell cyst to 16-cell cyst and to the egg chamber showing an increase  
852 in pseudouridine levels (n = 10, \* P < 0.0313, \*\*\* p < 0.001).  
853 (G) Statistics shown from the 2B-region of *UAS-Dcr2;nosGAL4* and *Nop10* or *Nop60* depleted  
854 germaria. Loss of H/ACA box members led to a significant reduction in germline pseudouridine  
855 levels when normalized to soma (n = 10, \*\*\* p < 0.001).  
856



Supplemental Figure 4

857  
858 **Supplemental 4: The H/ACA box is required in the cyst stages**  
859 (A-C) *bamGAL4* (driver control) germaria (A) and germline depletion of the *Nop10* (B) or *Nop60B*  
860 (C) stained with anti-1B1 (magenta) and anti-Vasa (green). Scale bar for all images is 20  $\mu$ m.

861 (D) Quantification of oogenesis defect phenotypes per genotype showing a cyst differentiation  
862 defect. Statistical analysis performed with Fisher's exact test (n = 32 for all, \*\*\* p<0.0001).  
863 (E) *bamGAL4* (driver control) germaria and (F and G) germline depletion of *Nop10* and *Nop60B*  
864 stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-Rbfox1 (green). Rbfox1 is shown in  
865 gray scale (E', F' and G'). Scale bar for all images is 20  $\mu$ m for all images. Yellow dotted line  
866 outlined cysts.  
867 (H) Quantification of Rbfox1 levels showing a reduction in Rbfox1 levels in *Nop10* and *Nop60B*  
868 depleted germaria. Statistical analysis performed with Fisher's exact test (n = 10 each, \*\*\*  
869 p<0.0001).  
870

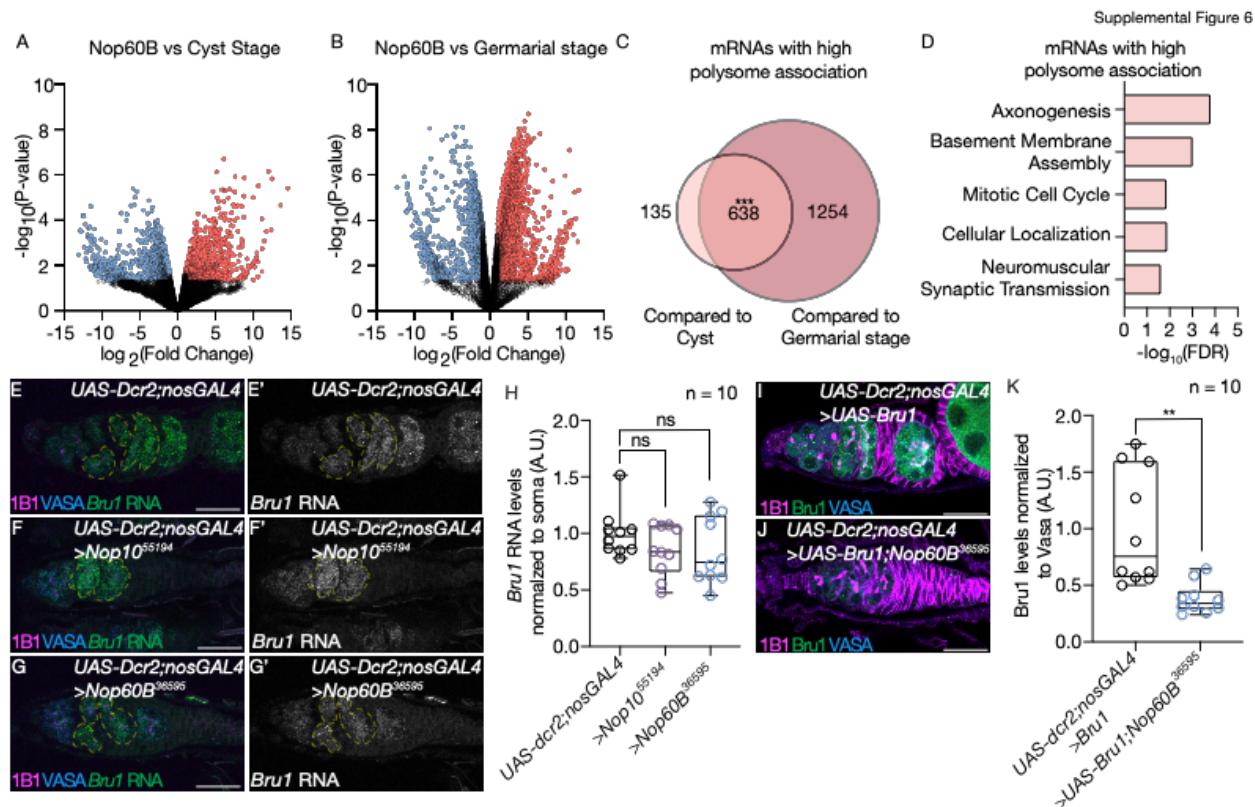


Supplemental Figure 5

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Supplemental 5: The H/ACA box deposits pseudouridine on rRNA and is required for proper ribosome biogenesis

874 (A) *Nop60B::GFP* germanium stained with Fibrillarin (magenta) and GFP (green). Fibrillarin is  
875 shown in gray scale (A'). Scale bar for all images is 20  $\mu$ m.  
876 (B) Fluorescence intensity plot generated from a box of averaged pixels centered around the  
877 punctate of Fibrillarin in the white box. R values denote Spearman correlation coefficients  
878 between GFP and Fibrillarin from plot profiles generated using Fiji, taken from the nucleolus  
879 denoted by the yellow box.  
880 (C) *UAS-Dcr2;nosGAL4* (driver control) germania and (D and E) germline depletion of *Nop10* and  
881 *Nop60B* stained with fibrillarin (magenta) and Vasa (green). Fibrillarin is shown in gray scale (C',  
882 D' and E'). Scale bar for all images is 20  $\mu$ m.  
883 (F) Quantification of nucleolar volume in the cysts stages per genotype showing an increased  
884 nucleolar size with *Nop10* and *Nop60B* depletion when compared to control. Statistics performed  
885 were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA (n = 20 each, \*\*\*  
886 p<0.0001).  
887 (G) Germania of *RpS5b-HA* stained with anti-1B1 (magenta) and anti-HA (green). HA is shown in  
888 gray scale (G').  
889 (H) Agarose gel of control lysate showing enrichment of rRNA (red arrow) in the input and IP lane  
890 but not in negative control (IgG).  
891 (I) Western blot analysis of ribosomal pulldowns probing for HA and RPL26 in input, IgG and  
892 pulldown samples. HA and RPL26 are present in both the input and pulldown lane but not the IgG  
893 showing successful pulldown of large and small ribosomal proteins.  
894 (J) Polysomes traces for YWT (*UAS-Dcr2;nosGAL4*) (black) and *Nop60B* (red) depleted germania.  
895 *Nop60B* is required for proper ribosome biogenesis as traces show that loss of *Nop60B* results in  
896 40S and 60S defects as well as loss of polysomes when compared to control.  
897



898

899 **Supplemental 6: The H/ACA box is required for translation of meiotic mRNAs**

900 (A) Volcano plot of *Nop60B* depleted germaria vs cyst stages (heat shock) with  $\log_2$ (fold change) 901 on x-axis and  $-\log_{10}(P\text{-value})$  on the y-axis. Blue points represent mRNAs that have lower 902 association with the polysomes and red points represent mRNAs with high polysome association 903 ( $n = 2$ , targets identified as 2-fold cutoff).

904 (B) Volcano plot of *Nop60B* depleted germaria vs germarial stages (YWT or *UAS-Dcr2;nosGAL4*) 905 with  $\log_2$ (fold change) on x-axis and  $-\log_{10}(P\text{-value})$  on the y-axis. Blue points represent mRNAs 906 that have lower association with the polysomes and red points represent mRNAs with high 907 polysome association ( $n = 2$ , targets identified as 2-fold cutoff).

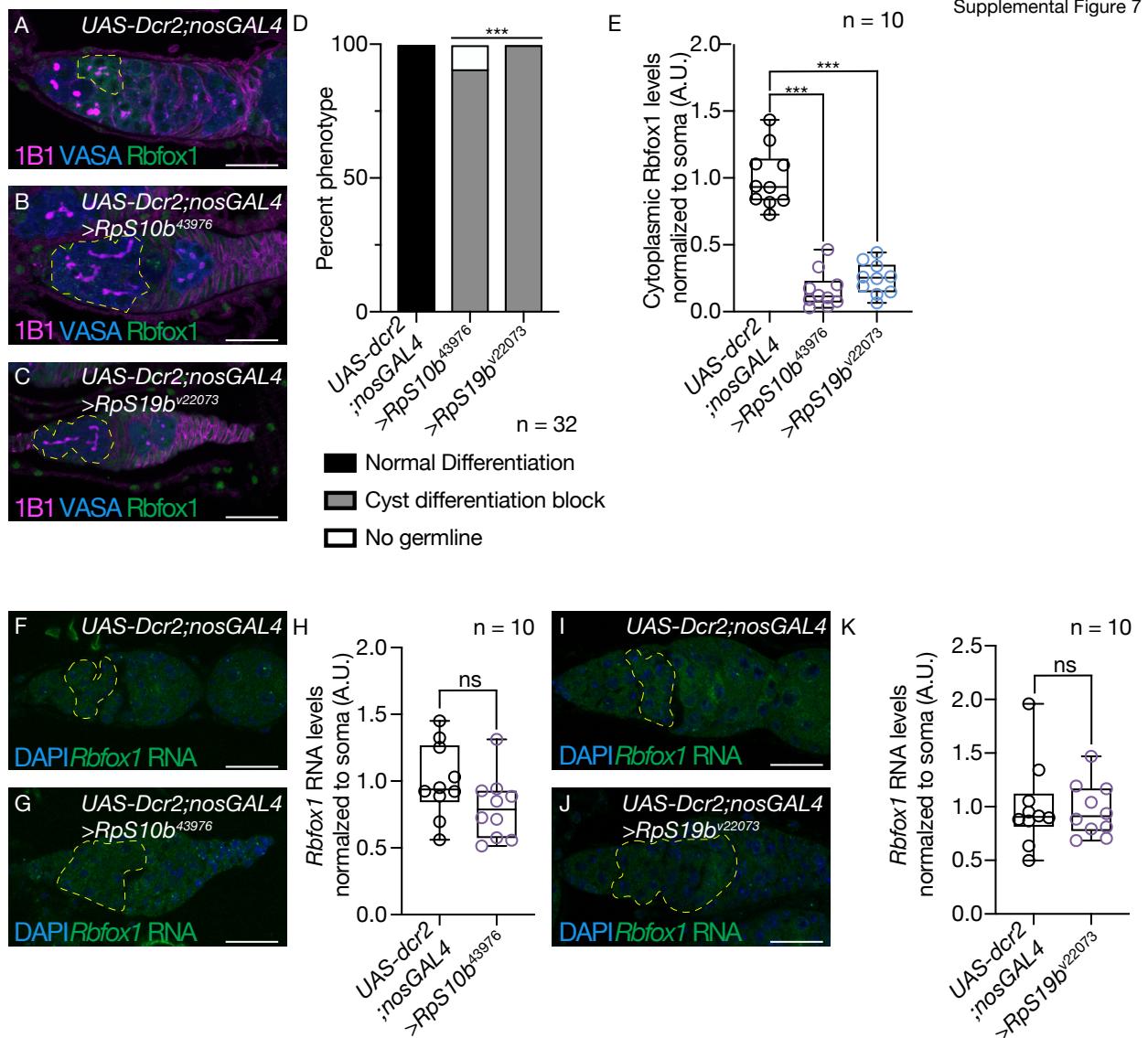
908 (C) Venn diagram illustrating overlap of *Nop60B*-polysome  $>2$  fold more association upon loss 909 of *Nop60B* (significance to low to compute in RStudio using Hypergeometric Test). Controls were 910 cysts, enriched through heat shock, and germarial stages (YWT or *UAS-Dcr2;nosGAL4*)

911 (D) Significant biological process GO terms of shared highly associated mRNAs in 912 ovaries depleted of *Nop60B* compared to control sets, showing an enrichment for mRNAs 913 associated with mitotic cell cycle.

914 (E-G) *In situ* hybridization to *Bru1* RNA (green) together with anti-1B1 (magenta) and anti-Vasa 915 (blue) staining in *UAS-Dcr2;nosGAL4* (driver control) germaria (E) and germline depleted of 916 *Nop10* (F) and *Nop60B* (G). *Bru1* RNA is shown in gray scale (E', F', G'). Scale bar for all images 917 is 20  $\mu\text{m}$ . Yellow dotted line outlines *bru1* RNA.

918 (H) Quantification of *Bru1* RNA levels in germline depleted of varying members of *Nop10* and 919 *Nop60B* normalized to soma showing no significant change in *Bru1* RNA levels. Statistics 920 performed were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA ( $n = 10$  921 each, not significant,  $p=0.3606$  and  $p=0.3752$  respectively).

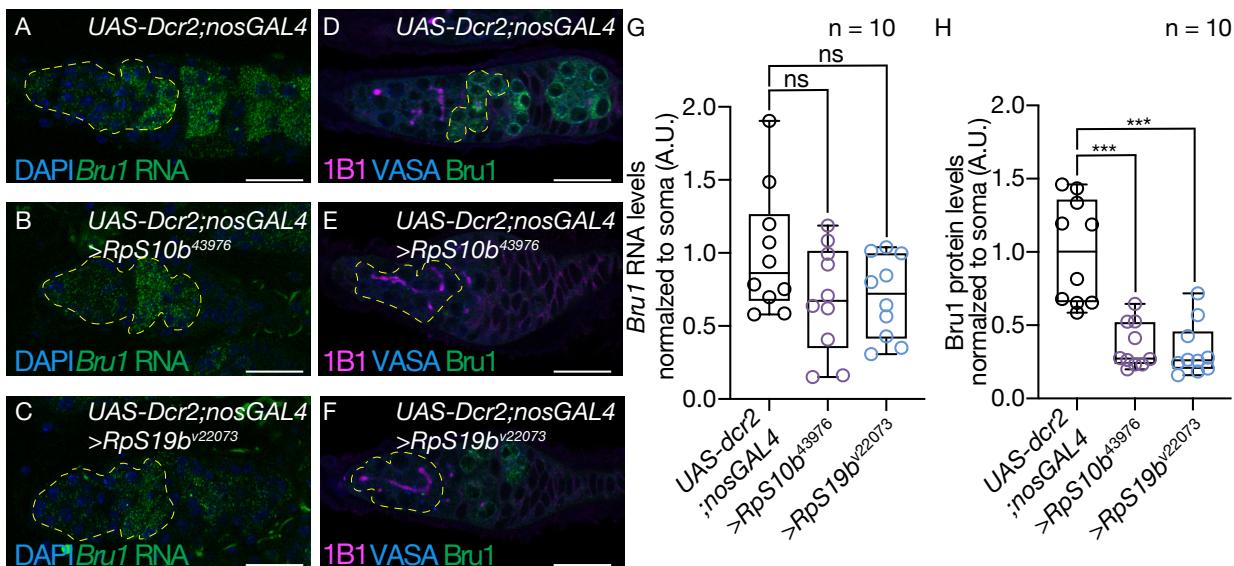
922 (I) Germaria of *UAS-Dcr2;nosGAL4* (driver control) overexpressing Bru1 and (J) germline  
 923 depleted of *Nop60B* overexpressing Bru1. Scale bar for all images is 20  $\mu$ m.  
 924 (K) Quantification of Bru1 levels in control vs germline depleted of *Nop60B* normalized to Vasa  
 925 show a reduction in Bru1 levels. Statistics performed were unpaired t-test (n = 10 each, \*\*  
 926 p=.0015).  
 927



928  
 929 **Supplemental 7: Ribosomal paralogs are required for Rbfox1 translation**  
 930 (A-C) *UAS-Dcr2;nosGAL4* (driver control) germaria (B) germline depletion of *RpS10b* (B) or  
 931 *RpS19b* (C) stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-Rbfox1 (green). Scale bar  
 932 for all images is 20  $\mu$ m. Yellow dotted lines outline cysts.  
 933 (D) Quantification of oogenesis defect phenotypes per genotype. Knockdown of ribosomal  
 934 paralogs results in a cyst differentiation block. Statistical analysis performed with Fisher's exact  
 935 test (n = 32 for all, \*\*\* p<0.0001).

936 (E) Quantification of cytoplasmic Rbfox1 levels normalized to soma in germline depletion of  
937 *RpS10b* and *RpS19b* showing that loss of ribosomal proteins results in lower Rbfox1 levels.  
938 Statistics performed were Dunnett's multiple comparisons test post-hoc test after one-way  
939 ANOVA (n = 10 each, \*\*\* p<0.0001).  
940 (F) In situ hybridization of *Rbfox1* RNA (green) and DAPI (blue) in *UAS-Dcr2;nosGAL4* (driver  
941 control) germlaria and (G) germline depleted of *RpS10b*. Scale bar for all images is 20  $\mu$ m. Yellow  
942 dotted line outlines *Rbfox1* RNA.  
943 (H) Quantification of *Rbfox1* RNA levels in germline depleted of *RpS10b* normalized to soma  
944 showing no significant differences in *Rbfox1* RNA levels. Statistics performed were unpaired t-  
945 test (n = 10 each, not significant, p=0.1006).  
946 (I) In situ hybridization of *Rbfox1* RNA (green) and DAPI staining (blue) in *UAS-Dcr2;nosGAL4*  
947 (driver control) germlaria and (J) germline depleted of *RpS19b*. Scale bar for all images is 20  $\mu$ m.  
948 Yellow dotted line outlines *Rbfox1* RNA.  
949 (K) Quantification of *Rbfox1* RNA levels in germline depleted of *RpS19b* normalized to soma  
950 showing no significant differences in *Rbfox1* RNA levels. Statistics performed were unpaired t-  
951 test (n = 10 each, not significant, p=0.8258).  
952

Supplemental Figure 8



953  
954 **Supplemental 8: Ribosomal paralogs are required for Bru1 translation**  
955 (A-C) In situ hybridization to *Bru1* RNA (green) and DAPI staining (blue) in *UAS-Dcr2;nosGAL4*  
956 (driver control) germlaria (A) and germline depleted of *RpS10b* (B) and *RpS19b* (C). Scale bar for  
957 all images is 20  $\mu$ m. Yellow dotted line outlines *bru1* RNA.  
958 (D-F) *UAS-Dcr2;nosGAL4* (driver control) germlaria (D) and germline depletion of *RpS10b* (E) and  
959 *RpS19b* (F) stained with anti-1B1 (magenta), anti-Vasa (blue) and anti-Bru1 (green). Scale bar  
960 for all images is 20  $\mu$ m. Yellow dotted line outlines cysts.  
961 (G) Quantification of *Bru1* RNA levels normalized to soma in germline depletion of *RpS10b* and  
962 *RpS19b* showing no significant differences in *Bru1* RNA levels with loss of ribosomal paralogs.

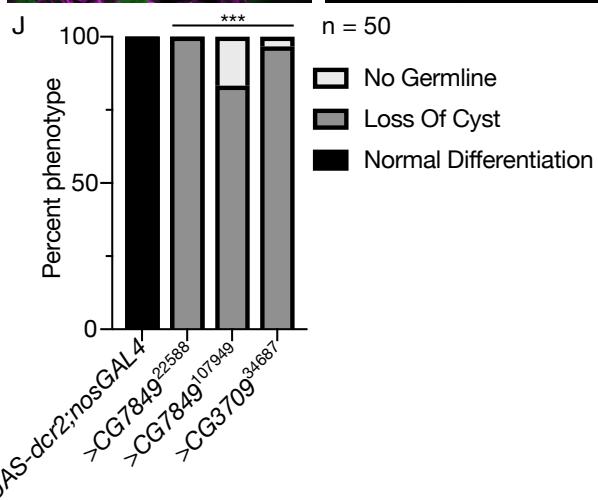
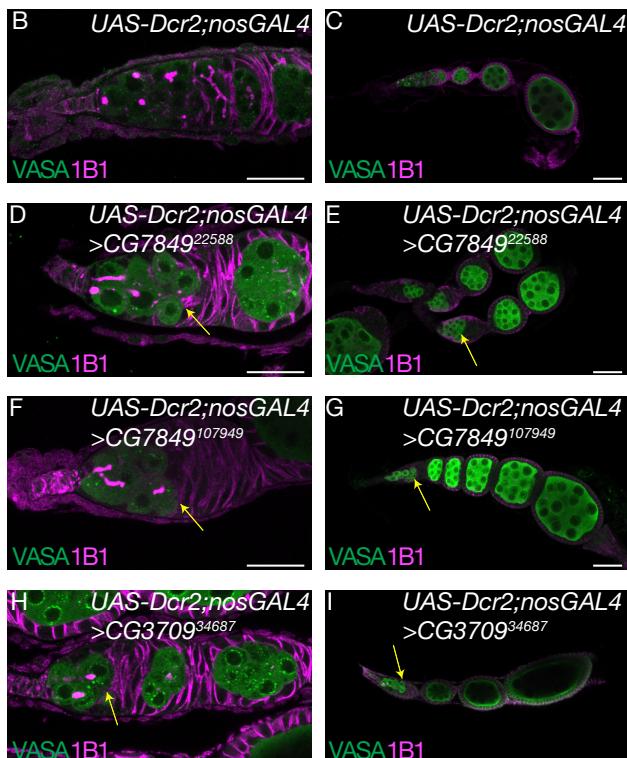
963 Statistics performed were Dunnett's multiple comparisons test post-hoc test after one-way  
964 ANOVA (n = 10 each, not significant, p=0.1149 and 0.1325, respectively).  
965 (H) Quantification of Bru1 protein levels normalized to soma in germline depletion of *RpS10b* and  
966 *RpS19b* showing that loss of ribosomal proteins results in lower bru1 levels. Statistics performed  
967 were Dunnett's multiple comparisons test post-hoc test after one-way ANOVA (n = 10 each, \*\*\*  
968 p<0.0001).  
969

### Supplemental Figure 9

A	Flybase symbol	Pseudouridine Synthase	Location
	CG7849	TruB/TruB2	Unknown tRNA or mRNA
	CG3709	Pus10	tRNA Uracil 55

## Germaria (40x)

### Ovariole (20x)

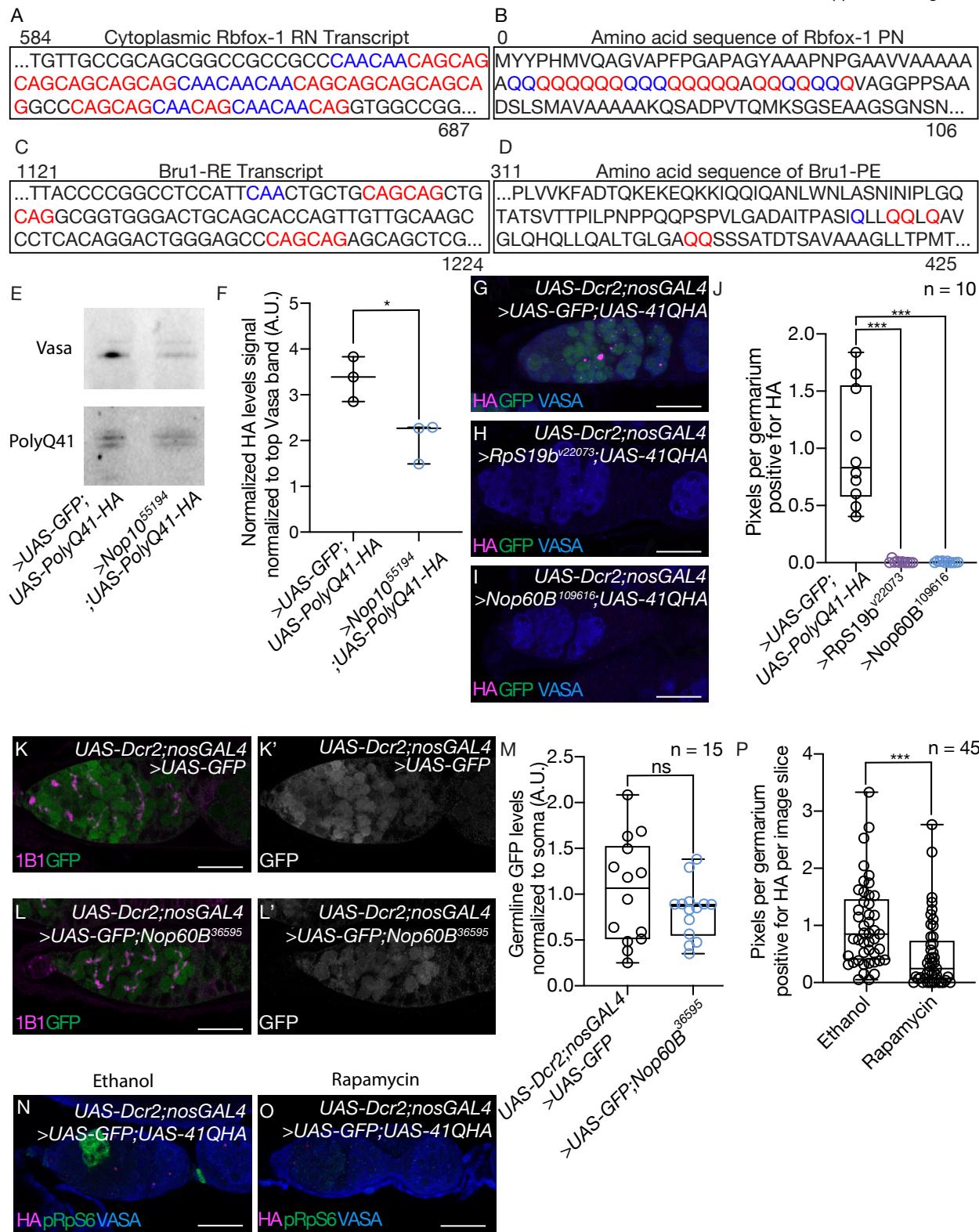


970  
971  
972

## Supplemental 9: tRNA pseudouridine synthases are required for differentiation but do not phenocopy loss of rRNA pseudouridine synthases

973 (A) Table of tRNA pseudouridine synthases and location of pseudouridine deposition found to  
974 have a differentiation defect.  
975 (B, C) Images of 40x *UAS-Dcr2;nosGAL4* (driver control) germarium (B) and 20x *UAS-*  
976 *dcr2;nosGAL4* (driver control) ovarioles (C) stained with anti-1B1 (magenta) and anti-Vasa  
977 (green).  
978 (D, E) Images at 40x (D) and 20x (E) of germarium where CG7849 is depleted in the germline  
979 and stained with anti-1B1 (magenta) and anti-Vasa (green).  
980 (F, G) Images at 40x (F) and 20x (G) of germarium using a second RNAi line to deplete CG7849  
981 in the germline and stained with anti-1B1 (magenta) and anti-Vasa (green).  
982 (H, I) Images at 40x (H) and 20x (I) of germarium where CG3709 is depleted in the germline and  
983 stained with anti-1B1 (magenta) and anti-Vasa (green). Yellow arrow points to region where cysts  
984 are lost in all 20x images. Scale bar for all images is 20  $\mu$ m.  
985 (J) Quantification of oogenesis defect phenotypes in tRNA pseudouridine synthase germline  
986 knockdowns resulting in loss of cyst defect. Statistical analysis performed with Fisher's exact test  
987 ( $n = 50$  each, \*\*\*  $p < 0.0001$ ).  
988

Supplemental Figure 10



991 (A) mRNA sequence of cytoplasmic Rbfox1-RN with glutamine (Q) codons CAA in blue and CAG  
992 in red. The transcript contains a large region of repeating CAA and CAG.  
993 (B) Protein sequence of Rbfox1-PN. Blue letters represent a Q encoded by CAA while red letters  
994 represent Q encoded by CAG. The transcript contains a large polyQ region.  
995 (C) mRNA sequence of Bru1-RE with CAA in red and CAG in blue. The transcript contains a large  
996 region of repeating CAA and CAG.  
997 (D) Protein sequence of Bru1-PE with Q encoded by CAA in blue, while red letter represent Q  
998 that corresponds to the codon CAG. The transcript contains a large polyQ region Q.  
999 (E) Western blot analysis of poly41Q-HA reporter driven in control and *Nop10* depleted germlaria  
1000 driven by *UAS-Dcr2;nosGAL4*. Western was probed with HA to detect polyQ protein. Vasa was  
1001 probed for normalization of germline.  
1002 (F) The level of HA (polyQ-HA) in ovary are significantly reduced upon germline knockdown of  
1003 *Nop10*. Protein immunoblots for HA were performed using extracts from whole ovaries. The signal  
1004 ratio between the HA and the upper Vasa band were used to quantitate and normalize the amount  
1005 of germline. The ratio is expressed in arbitrary units (A.U.). The results of each independent  
1006 experiment are plotted. Statistics performed were unpaired t-test (n = 3, \* p=.0253).  
1007 (G) Control confocal image of poly41Q-HA reporter driven in *UAS-Dcr2;nosGAL4* and germlaria  
1008 depleted of *RpS19b* and *Nop60B* (H and I) stained with anti-HA (magenta), anti-GFP (green) and  
1009 anti-Vasa (blue). Scale bar for all images is 20  $\mu$ m.  
1010 (J) Quantification of percent of pixels per area of HA in control vs germline depleted of *RpS19b*  
1011 and *Nop60B* showing a reduction in HA signal. Statistics performed were unpaired t-test (n = 10  
1012 each, \*\*\* p=0.0001).  
1013 (K, L) UAS-GFP driven by *UAS-Dcr2;nosGAL4* in control germlaria (K) and in germlaria depleted  
1014 of *Nop60B* (L), stained with anti-1B1 (magenta) and anti-GFP (green). GFP is shown in gray scale  
1015 (K' and L'). Scale bar for all images is 20  $\mu$ m.  
1016 (M) Quantitation of GFP levels in the cysts stages normalized to somatic background per  
1017 genotype. There is no significant difference in GFP levels between control germlaria and *Nop60B*  
1018 depleted germlaria. The results of each independent experiment are plotted. Statistics performed  
1019 were unpaired t-test (n = 15, not significant, p=0.2187).  
1020 (N, O) Confocal image of poly41Q-HA reporter driven by *UAS-Dcr2;nosGAL4* in mock-treated (N)  
1021 and rapamycin-treated (O) ovaries, stained with anti-HA (magenta), anti-pRpS6 (green) and anti-  
1022 Vasa (blue). Scale bar for all images is 20  $\mu$ m.  
1023 (P) Quantification of percent of pixels of HA per area in mock- and rapamycin-treated flies showing  
1024 a reduction in HA signal with rapamycin treatment. Statistics performed were unpaired t-test (n =  
1025 45 slices quantified for each, \*\*\* p<0.0005).  
1026  
1027 **Table 1:** (A) PTM code for the modifications identified. The 1<sup>st</sup> column represents the Modomics  
1028 code, the 2<sup>nd</sup> column represents the PTM name and the 3<sup>rd</sup> column the shortened modification  
1029 name. (B) Summary of RNA PTMs profiles obtained from GSCs, GSC daughters, cysts (early  
1030 cysts), young wild type (later cysts and early egg chambers) and wild type (late-stage egg  
1031 chambers). Each value represents the average and standard deviation of the respective relative  
1032 abundances (AvP%, see Methods). A different shade of color was assigned only if the RNA PTMs  
1033 relative abundance was statistically different from that of the GSCs input reference (1st column)  
1034 with a p value not exceeding 0.05.

1035  
1036 **Table 2:** (A) Excel spreadsheet of the RNA modification screen that contains the gene names,  
1037 stock numbers, type of modification and phenotype. The raw number of germaria were counted.  
1038 (B) The RNA modification screen represented as percent phenotypes.  
1039  
1040 **Table 3:** Summary of PTM profiles obtained. Each value represents the average and standard  
1041 deviation of the respective relative abundances (AvP%, see Methods). A different shade of color  
1042 was assigned only if the RNA PTMs relative abundance was statistically different from that of the  
1043 cysts input reference (1st column) with a p value not exceeding 0.05.  
1044  
1045 **Table 4:** Spreadsheet of mRNA targets identified from pull-down utilizing pseudouridine antibody  
1046 with a 2-fold cut off. (A) Genes that were lower than 2-fold enriched (B) genes that were higher  
1047 than 2-fold enriched and (C) fold-enrichment values for all genes.  
1048  
1049 **Table 5:** (A) MEME discriminative mode motif enrichment output of the 5' UTR, CDS and 3' UTR  
1050 of genes that are lowly associated with the ribosome in germaria depleted of Nop60B. E-value,  
1051 sites and width are provided for each identified motif. (B) MEME discriminative mode motif  
1052 enrichment output of the 5' UTR, CDS and 3' UTR of genes highly associated polysome in  
1053 germaria depleted of Nop60B depletion. E-value, sites and width are provided for each identified  
1054 motif.  
1055  
1056 **Table 6:** (A) Correlation plots comparing Ribo-Seq datasets showing high reproducibility between  
1057 libraries. (B) Column A: mRNA targets identified by Ribo-Seq that contain the CAG motif. Column  
1058 B: mRNAs containing a strict repeating CAG (no interruptions). Column C: locations of the CAG  
1059 motif. Column D: length of the longest CAG repeat present in the mRNA or if there are other  
1060 amino acid repeats present.  
1061  
1062 **Table 7:** (A) Find Individual Motif Occurrences (FIMO) output of QQQQQ motif search in genes  
1063 that were lowly associated with the polysome in Nop60B depleted germaria. Representative of  
1064 181 unique genes that significantly contain a motif resembling QQQQQ. (B) All transcripts from  
1065 the Find Individual Motif Occurrences (FIMO) output of QQQQQ motif search in genes lowly  
1066 associated with the polysome in Nop60B depleted germaria. Also provided are the p-value and  
1067 matched motif sequences in each transcript.  
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