# Title: Sleep and memory consolidation are linked by RNA processing genes in the *Drosophila* mushroom body

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**Abstract**

Memory consolidation in *Drosophila* can be sleep-dependent or sleep-independent, depending on the availability of food. Different regions of the mushroom body (MB) mediate these two mechanisms, with the anterior posterior (ap) alpha′/beta′ (α′/β′) neurons required for sleep-dependent memory consolidation in flies that are fed after training. These neurons are also involved in the increase of sleep after training, suggesting a coupling of sleep and memory. To better understand the mechanisms underlying sleep and memory consolidation initiation, we analyzed the transcriptome of ap α′/β′ neurons one hour after appetitive memory conditioning. A small number of genes were differentially expressed specifically in flies fed after training, but not in trained and starved flies or untrained flies. Knockdown of each of these differentially expressed genes in the ap α′/β′ neurons revealed multiple genes that affect sleep, with notable effects observed for Polr1F and Regnase-1, both of which decrease in expression after conditioning. Knockdown of Polr1F, a regulator of ribosome RNA transcription, in adult flies promotes sleep and increases pre-ribosome RNA expression as well as overall translation, supporting a function for Polr1F downregulation in memory consolidation. Conversely, knockdown of Regnase-1, an mRNA decay protein localized to the ribosome, reduces sleep. Given that Regnase-1 knockdown in ap α′/β′ neurons affects both sleep-dependent and sleep-independent memory, as well as short-term memory, Regnase-1 likely has an early role in the learning process, which may

obscure a later function for its downregulation during sleep-dependent memory. These findings indicate that changes in RNA processing play a crucial role in triggering post-training sleep and memory consolidation.

## Background

Sleep is an optimized state for memory consolidation compared to wake (Rasch and Born, 2013). Indeed, sleep is thought to reorganize and strengthen the neural connections required for novel memory formation and long-term memory consolidation, and sleep disruption leads to impaired memory consolidation (Roselli et al., 2021). Beneficial effects of sleep on memory have also been attributed to post-learning neuronal reactivation (Wagner et al., 2007; Dag et al., 2019).

We recently demonstrated that *Drosophila* switch between sleep-dependent and sleep-independent memory consolidation based on food availability (Chouhan et al., 2021). Flies that are fed after appetitive conditioning show sleep-dependent memory consolidation, which is mediated by the anterior posterior (ap) α'/β' neurons of the mushroom body. On the other hand, flies starved after training display sleep-independent memory mediated by medial (m) neurons of the α'/β' lobes. Neurotransmission from ap α′/β′ neurons in the first 4 hours after training in the fed flies is not only required for long term memory but also increased sleep post-training. Indeed, activation of ap α′/β′ neurons promotes baseline sleep, supporting the idea that post training sleep is triggered by the same neurons that are required for sleep-dependent

58 memory consolidation. However, how sleep and memory consolidation are coordinated
59 in ap α′/β′ neurons in this time window is not understood.

60 Since both post-training sleep and memory consolidation occur rapidly within a
61 few hours after training, we investigated transient gene expression changes in the ap
62 α′/β′ neurons after training to elucidate the interplay of sleep and memory consolidation.
63 Here, we profiled the transcriptome of ap α′/β′ neurons 1 hour after flies were fed and
64 trained, as well as under control conditions in which flies were starved and trained or fed
65 and untrained. We knocked down the differentially expressed genes in trained and fed
66 flies and identified two RNA processing genes that affect sleep. Knockdown of one of
67 these, Polr1F, a regulator of ribosomal RNA synthesis, promotes sleep and translation,
68 both of which are required for consolidation of memory in trained and fed flies.
69 Knockdown of Polr1F does not affect memory, which is consistent with downregulation
70 of this gene during memory consolidation. Knockdown of Regnase-1, an mRNA decay
71 protein, reduces sleep and disrupts short- and long-term memory, suggesting an early
72 role, perhaps in learning. These results suggest that RNA processing is an important
73 mechanism linking sleep to memory.

## Results

### Transient transcriptome profiling of ap α′/β′ neurons after training

We previously demonstrated that flies fed after appetitive memory training exhibit increased sleep and form sleep-dependent memory, which is mediated by ap α′/β′ neurons of the mushroom body (MB) α'/β' lobes (Chouhan et al., 2021). To address the mechanisms that mediate increases in sleep and consolidation of memory in ap α′/β′ neurons, we assayed gene expression changes in ap α′/β′ neurons of flies fed after training in an appetitive conditioning paradigm. We crossed ap α′/β′ neuron driver *R35B12-Gal4* (BDSC #49822) with *UAS-nGFP* (BDSC #4775) to label all ap α′/β′ neurons with nuclear GFP and collected 5-7 day old F1 progeny, which were subjected to one of three different conditions: Trained-Fed, Trained-Starved, Untrained-Fed as illustrated (**Figure 1A**). The trained-starved flies served as controls for sleep-dependent changes, while flies that were fed but untrained served as controls for training-dependent changes. After one-hour, 50 mixed sex (25 for each sex) fly brains from each condition were dissected, and 500 GFP+ cells were sorted for bulk-RNA sequencing using the protocol described by Hongjie Li et al (Li et al., 2017).

Our analysis of the bulk RNA-seq data revealed that most genes are not altered in ap α′/β′ neurons after one hour, so there are no significant global changes observed among the three conditions. The correlation matrix calculated using the top 75% genes showed high similarity between samples, with most values exceeding 0.9 (**Figure 1 – source data**). Nonetheless, we did observe a small subset of genes that were rapidly responsive and differentially expressed between the groups (**Figure 1B**). Pathway Principal Component Analysis (PathwayPCA) of these differentially expressed genes

97 (DEGs), a statistical method to identify key patterns in gene expression data by
98 reducing data dimensionality, showed that genes contributing to PC1, which accounted
99 for 35% of the variance, largely encode proteins involved in transcription and
100 biosynthesis, including RNA biosynthesis processes (**Figure 1 – supplemental figure**
101 **1A**) (Odom et al., 2020). This suggests that the training and feeding paradigm
102 influenced transcription and RNA biosynthetic processes. Meanwhile, gene ontology
103 analysis of these DEGs, performed using DAVID Bioinformatics
104 (https://david.ncifcrf.gov/home.jsp), revealed significant enrichment in processes such
105 as protein unfolding/refolding and the stress response (**Figure 1 – supplemental figure**
106 **1B**) (Sherman et al., 2022).

107 Of all the DEGs across the three groups, we focused on 59 DEGs that were
108 significantly different in TrainedStarved vs. TrainedFed and UntrainedFed vs.
109 TrainedFed; of these, 56 were downregulated and only three were upregulated in the
110 Trained-Fed condition compared to the control conditions (**Figure 1B, D, Figure 1 –**
111 **source data**). Gene ontology (GO) analysis of these 59 genes using FlyEnrichr
112 (https://maayanlab.cloud/FlyEnrichr/) indicated that they encode cellular components of
113 the 90S preribosome, Cajal body, DNA-directed RNA polymerase complex, nuclear
114 euchromatin, and condensed chromosome, consistent with the PCA enrichment (**Figure**
115 **1C**) (Chen et al., 2013). Our transcriptome results suggest that ribosome biosynthesis
116 and transcription are the initial changes in ap α′/β′ neurons of trained and fed flies.

117

118 **Two genes expressed differentially in ap α′/β′ neurons of trained and fed flies**
119 **predominately affect sleep.**

120 To investigate if any of the 59 DEGs identified in the ap α′/β′ neurons of trained
121 and fed flies affect baseline sleep, we knocked down each of the genes using UAS-
122 RNAi lines and screened for their potential effects on sleep. We used the ap α′/β′
123 neuron constitutive driver R35B12-Gal4 line to drive the RNAi constructs and compared
124 the sleep patterns of knockdown flies with those of R35B12-Gal4 and UAS-RNAi control
125 flies (**Figure 2A, B, Figure 2 – source data**). Using a cutoff of a 200 min change in
126 sleep relative to each control group, we identified two genes, Polr1F and Regnase-1,
127 that showed significant effects on baseline sleep. Knockdown of Polr1F, a component of
128 RNA polymerase I complex, led to an increase in sleep. Although Polr1F has not been
129 extensively studied in *Drosophila* (Marygold et al., 2020), its human ortholog hRPA43 is
130 part of the multi-subunit protein complex Pol I that regulates the transcription of
131 ribosomal RNA (Beckouët et al., 2011). Knockdown of Regnase-1, an RNA-binding
132 protein that binds to mRNA undergoing active translation and promotes mRNA decay
133 via its ribonuclease activity, in ap α′/β′ neurons resulted in a reduction of both nighttime
134 and daytime sleep (**Figure 2C-F**). Further analysis of sleep architecture revealed that
135 knockdown of Polr1F and Regnase-1 did not significantly impact the total activity of
136 flies, while knockdown of either Polr1F and Regnase-1 resulted in increased and
137 decreased average length of sleep episodes, respectively (**Figure 2 – supplemental**
138 **figure1A-F**).

139 Given that these two genes reduce expression rapidly in response to training
140 under fed conditions, we next used the inducible pan-neuronal GeneSwitch driver *nSyb-*
141 *GeneSwitch (GS)* to determine if restricting knockdown of these two genes to the adult
142 stage recapitulates changes in sleep and/or memory. RU486 (mifepristone) was added

143 to normal fly food and transgene expression is induced when flies are loaded into
144 *Drosophila* Activity Monitor (DAM) glass tubes (Robles-Murguia et al., 2019). We
145 crossed the *nSyb-GS* flies with Polr1F or Regnase-1 RNAi flies and transferred the
146 adult F1 progeny to RU486 tubes to induce expression of the RNAi 3-5 hours before
147 dusk, and continuously monitored sleep for 5 days. Knockdown of Polr1F resulted in
148 immediate inactivity and sleep (**Figure 3A-C**). However, with knockdown of Regnase-1,
149 immediate changes in sleep were not noted (**Figure 3 – supplemental figure 2A-C**).
150 This could be due to potential leakiness of the nSyb-GS, such that it reduced sleep
151 even without RU486 treatment, coupled with the fact that sleep before dusk is already
152 quite low, potentially hindering detection of further sleep reduction. We also analyzed
153 sleep for the next 3 consecutive days from day 3 to day 5 and found that constitutive
154 pan-neuronal Polr1F knockdown increased sleep while Regnase-1 knockdown seemed
155 to have no effect, again perhaps due to the leakiness of *nSyb-GS.* However, it is also
156 possible that Regnase-1 affects sleep during developmental stages or acts differently in
157 different brain areas (**Figure 3D, E; Figure 3 – supplemental figure 2D, E**). In general,
158 these results indicate that adult specific knockdown of Polr1F promotes sleep while
159 brain-wide adult-specific knockdown of Regnase-1 has limited effect on sleep.

160 To further assess the adult-specific sleep function of both genes in ap α′/β′
161 neurons, we coupled the R35B12-Gal4 driver to tubulin-Gal80$^{ts}$, which allows
162 temperature-dependent expression of Gal4, and crossed it with the UAS-Polr1F$^{RNAi}$ and
163 UAS-Regnase-1$^{RNAi}$ lines. We confirmed that Polr1F RNAi promotes both transient and
164 chronic sleep when flies were transferred from permissive to restrictive temperatures
165 during the adult stages (**Figure 3 – supplemental figure 1**). Conversely, Regnase-1

166 RNAi showed no effect on sleep in the adult stage, consistent with our nSyb-GS
167 experiments, suggesting that the Regnase-1 RNAi sleep effect is likley developmental
168 (**Figure 3 – supplemental figure 3**). We also analyzed the daily peak activity of the
169 flies to determine if either Polr1F RNAi or Regnase-1 RNAi affected activity. We found
170 that neither gene affected peak activity, suggesting that both genes influence sleep but
171 not activity (**Figure 3 -supplemental figure4**).

172 **Knockdown of Regnase-1 affects memory**

173 We next evaluated the impact of Polr1F and Regnase-1 knockdown on memory
174 consolidation using our olfactory conditioning paradigm (**Figure 4A**). Starved flies were
175 subjected to training to associate an odor with a reward, and then post-training, they
176 were either kept on food vials for sleep-dependent memory consolidation or kept
177 starved to promote sleep-independent memory consolidation. Memory tests were
178 conducted 24 hours after training for starved flies, while fed flies were restarved for 42
179 hours before testing, as starvation is necessary for memory retrieval (Krashes and
180 Waddell, 2008). Efficacy of the RNAi line used above was verified by qPCR
181 experiments (**Figure 4 -supplemental figure 1A**). We observed that constitutive
182 knockdown of Polr1F in ap α′/β′ neurons did not affect sleep-dependent or sleep-
183 independent memory as memory performance was comparable to that of genetic
184 controls (**Figure 4B-C**). These results were consistent with the fact that Polr1F levels
185 typically decrease during memory consolidation. Monitoring of sleep from ZT8 to ZT12
186 after training at zeitgeber time (ZT) 6 showed that the post-training increase in sleep
187 was also not affected by Polr1F knockdown in ap α′/β′ neurons (**Figure 4E**), suggesting

188 that acute downregulation of Polr1F may not be essential to the post-training increased
189 sleep.

190 On the other hand, constitutive Regnase-1 knockdown in ap α′/β′ neurons
191 resulted in a significant decrease in long-term memory performance in both fed and
192 starved flies and eliminated the increase in post-training sleep (**Figure 4B-E**). These
193 findings suggested that Regnase-1 expression in ap α′/β′ neurons is necessary for both
194 sleep-dependent and sleep-independent memory consolidation. It is also possible that
195 disruption of Regnase-1 in ap α′/β′ neurons affects learning, short-term memory
196 formation, or appetitive memory retrieval (Aso et al., 2014; Shyu et al., 2019; Rutherford
197 et al., 2023). Indeed, short-term memory tests confirmed that Regnase-1 knockdown
198 flies performed significantly worse than control flies (**Figure 4F**), indicating that
199 Regnase-1 acts early in the learning/memory process, which might explain its effect on
200 sleep-dependent and sleep-independent memory. We also tested Regnase-1
201 overexpression for effects on sleep, and found that it does not affect sleep (**Figure 4 -**
202 **supplemental figure 1B**) (Zhu et al., 2019).

203

204 **Knockdown of Polr1F promotes translation**

205 Since Polr1F knockdown promotes sleep and a 22 amino acid peptide within
206 Polr1F inhibits ribosomal DNA transcription (Rothblum et al., 2014), we predicted that
207 Polr1F acted as a suppressor for ribosomal DNA transcription, and thus knockdown of
208 Polr1F would enhance the transcription and translation of ribosomal RNA and thereby
209 overall protein synthesis. As Regnase-1 is thought to promote decay of mRNAs

210 undergoing translation, its knockdown, or its downregulation following training, might
211 also be expected to promote translation, or at least the translation of its target mRNAs.
212 The role of protein synthesis in long-term memory consolidation is well-established
213 across organisms (Alberini and Kandel, 2015), and nascent rRNA synthesis was also
214 shown to be induced by training and required for memory consolidation in mouse (Allen
215 et al., 2018). We thus used real-time qPCR of total RNA extracted from vehicle control
216 and RU-treated fly brains to measure how precursor ribosomal RNA (Pre-rRNA) is
217 affected by knockdown of Polr1F. We found that the pre-rRNA level increased
218 significantly in the RU486 induction (RU+) group compared with vehicle control (RU-)
219 group for *nSyb-GS>polr1F RNAi* flies (**Figure 5A**), indicating Polr1F knockdown results
220 in higher levels of pre-rRNA, which is consistent with studies of Polr1F homologs in
221 yeast cells (Thuriaux et al., 1995; Rothblum et al., 2014).

222 The increasing ribosomal RNA should help translation and so we also used
223 incorporation of puromycin into newly synthesized peptides as an estimate of translation
224 after inducing pan-neuronal knockdown of Polr1F. We observed significantly higher
225 levels of anti-puromycin GFP fluorescence when Porl1F was knockdown pan-neuronally
226 (RU+) compared to the control (RU-) group (**Figure 5B**), indicating that Polr1F
227 suppresses translation, probably by suppressing ribosomal RNA transcription
228 (Rothblum et al., 2014). Thus, it may need to be downregulated after training to support
229 translation and memory. Given that knockdown of Porl1F enhances rRNA synthesis and
230 sleep, there is a question as to whether global alterations in rRNA synthesis impact
231 sleep. However, feeding of an rRNA inhibitor (CX-5461) failed to produce rapid sleep
232 phenotypes in fed or starved flies (**Figure 5 – supplemental figure 1**), suggesting that

233 rRNA synthesis may not directly influence sleep. Using puromycin to address effects of
234 Regnase-1 knockdown on translation revealed an insignificant slight increase in
235 translation (data not shown); given that Regnase-1 may specifically affect pre-existing
236 translationally active mRNA or may act only on specific target mRNAs, its effects on de
237 novo translation may not be obvious.

238 **Discussion**

239 The anterior-posterior (ap) α′/β′ neurons of the mushroom body make critical and
240 privileged contributions to sleep-dependent memory consolidation and post-training
241 sleep (Krashes et al., 2007; Chouhan et al., 2021), but the mechanisms that link
242 memory and sleep in these neurons are not known. To address this gap, we conducted
243 transcriptomic analysis of ap α′/β′ neurons from trained and fed flies to identify genes
244 that change rapidly under conditions that drive sleep-dependent memory. By uncovering
245 two RNA processing genes involved in memory and sleep, our transcriptome profiling of
246 ap α′/β′ neurons suggests that genes regulating rRNA transcription and translation are
247 altered in the context of sleep-dependent memory consolidation.
248

249 **RNA processing genes mediate sleep-dependent memory consolidation and**
250 **sleep.**

251 Many of the 59 DEGs we identified are implicated in RNA processing. Of these,
252 Polr1F and CG11920 affect ribosomal RNA processing, CG5654 is predicted to be part
253 of the 90S pre-ribosome and involved in endonucleoytic cleavages (Herold et al., 2009),
254 WDR79 encodes a small Cajal body specific RNA binding protein, Nup133 encodes a
255 component of nuclear pore complex, Regnase-1 degrades mRNA, and CG18011,
256 CG17568, Koko, CG11398 and Meics are all involved in RNA polymerase II-specific
257 transcription (Di Giorgio et al., 2017; Rogg et al., 2022). The enrichment of RNA
258 processing and translation genes is consistent with the notion that memory
259 consolidation requires transcription and translation (Seibt and Frank, 2012; Alberini and

260 Kandel, 2015). More importantly, our findings here indicate that alterations of some of
261 RNA processing genes may impact sleep as well.
262
263 **Polr1F regulates ribosome RNA synthesis and sleep**
264 Learning-induced changes in gene expression in memory-related neurons are
265 often critical for long-term memory formation (Cavallaro et al., 2002; Hoedjes et al.,
266 2015; Tadi et al., 2015). Our findings with Polr1F implicate changes in Pol I transcription
267 during sleep-dependent memory. Polr1F(Rpa43) is predicted to be part of the RNA
268 polymerase I complex and is involved in DNA-dependent RNA polymerase
269 activity/rDNA transcription in yeast, especially for the initiation of ribosomal RNA
270 (Beckouët et al., 2011; Marygold et al., 2020). As suggested by our data, Polr1F has an
271 inhibitory role in the RNA polymerase I complex, which is also consistent with the
272 aforementioned study that found a 22 amino acid peptide within Polr1F can inhibit rDNA
273 transcription (Rothblum et al., 2014). Based upon our finding that inducible knockdown
274 of Polr1F rapidly promotes translation, it is likely that the rapid and dramatic decline of
275 Polr1F after training in fed flies serves to increase *de novo* ribosome RNA synthesis.
276 This supports the report that ribosomal RNA is induced by learning and required for
277 memory consolidation in mice (Allen et al., 2018). While we do not know how
278 knockdown of Polr1F promotes sleep, an attractive possibility is that higher translation is
279 a result of elevated sleep. Sleep is thought to promote translation and its role in memory
280 consolidation in fed flies could be related to its effect on translation (Seibt and Frank,
281 2012; Chouhan et al., 2021). Alternatively, increased translation or rRNA synthesis
282 could promote sleep. However, translation is typically thought of as a consequence of

283 sleep rather than a cause (Zimmerman et al., 2006). Also, rRNA transcription rates
284 remain constant throughout the day in the liver (Sinturel et al., 2017), although it is still
285 possible that these rates vary in particular regions of the brain and affect sleep. The role
286 of rRNA synthesis in *Drosophila* learning and memory has barely been explored, but our
287 work, together with that of Allen et al. (2018), indicates that the well-known requirement
288 for *de novo* protein synthesis during long-term memory consolidation (Jarome and
289 Helmstetter, 2014) includes increased synthesis of ribosomal RNA and protein.
290

291 **Regnase-1 inactivation may affect memory through effects on translation**

292 The role of RNA binding protein Regnase-1 in the innate immune response has
293 been extensively studied (Mino et al., 2015; Mao et al., 2017; Wei et al., 2019).
294 However, our study sheds light on a novel function of Regase-1 in ap α′/β′ neurons on
295 sleep and memory. Regnase-1 is an anti-inflammatory enzyme that inhibits mRNA
296 translation during acute inflammatory responses. It localizes to the ribosomes on the
297 surface of the endoplasmic reticulum (ER) and binds to translationally active mRNAs
298 with specialized stem-loop structures at the 3′UTR (Uehata et al., 2013; Mino et al.,
299 2015). When phosphorylated, Regnase-1 is released from the ER (Tanaka et al., 2019).
300 Because functional Regnase-1 binds and degrades its bound mRNA, Regnase-1
301 inactivation leads to an increase of its target mRNA (Uehata et al., 2013). The target
302 mRNAs of Regnase-1 in immune cells encode proinflammatory cytokines, which can
303 then be expressed when Regnase-1 is inactivated. However, Regnase-1 has also been
304 reported to modulate cytokines and neuronal injury in the microglia in rats (Liu et al.,
305 2016). Regulation of Regnase-1 is usually rapid and transient, and its rapid response to

306 microenvironmental changes, different pathological states and stress is critical for
307 cellular adaption (Mao et al., 2017).

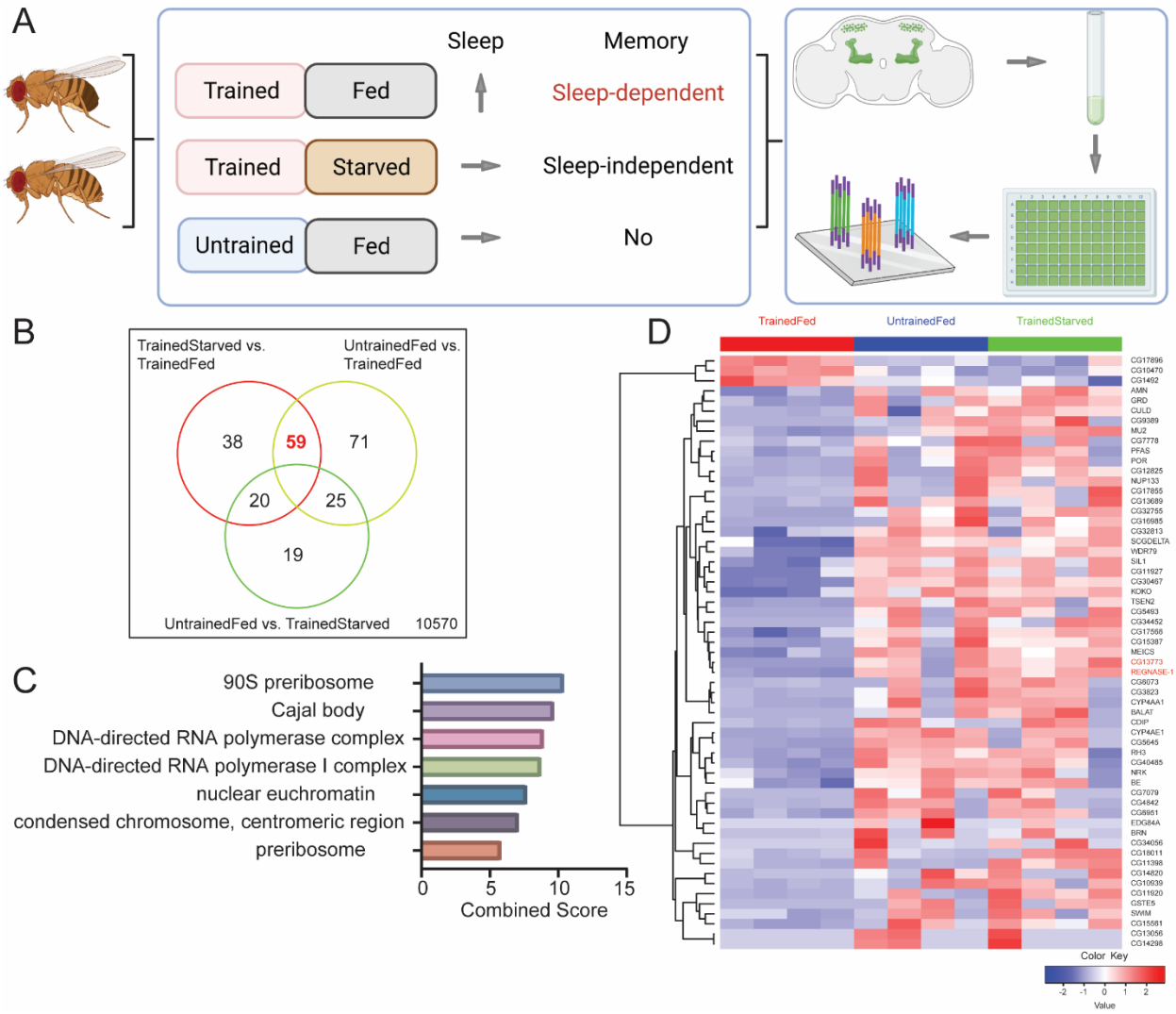
308 Our study reveals that the expression of Regnase-1 changes after training, and
309 constitutive downregulation of Regnase-1 in ap α′/β′ neurons reduce sleep and causes
310 deficits in learning and memory consolidation. We suggest that downregulation of
311 Regnase-1 following training in fed flies may be crucial for the promotion of long-term
312 memory, perhaps by promoting translation of specific transcripts. For instance,
313 downregulation of Regnase-1 after training may release a pool of mRNAs that are
314 normally targeted by it for decay and that are important for consolidation of memory. On
315 the other hand, we find that constitutive loss of Regnase-1 impairs sleep-independent
316 memory and also short-term memory, suggesting that it is required early in the learning
317 and memory process, likely for learning. Interestingly, constitutive knockdown of
318 Regnase-1 also reduces sleep and prevents sleep increase after training. However, our
319 adult specific knockdown of Regnase-1 does not affect sleep, suggesting that
320 expression of Regnase-1 during development is important for both memory and sleep.
321 Given that ap α′/β′ neurons have a role in several aspects of memory, consolidation as
322 well as short-term appetitive memory retrieval, how Regnase-1 acts within these
323 neurons to regulate memory clearly requires more investigation.

324 Our study of local molecular changes in ap α′/β′ neurons after training highlights
325 how ribosomal RNA transcription and mRNA translation work in concert during the
326 consolidation of sleep-dependent memory. Neurons are regulated to generate short
327 bursts of boosted transcription and boosted translation, which are required for new
328 protein synthesis. How sleep is involved in this boosted protein synthesis process is

329 unclear, but we suggest that sleep affects several steps of the central dogma, and that
330 deleterious consequences of sleep deprivation are mediated, at least in part, through
331 effects on rRNA transcription and translation. Overall, our findings indicate the
332 importance of maintaining RNA homeostasis for sleep and memory.

333

334 **Figures**




335

336 **Figure1. Differential gene expression after training in mushroom ap α′/β′ neurons.**

337 (A) 5-7 day old mixed sex *wCS* flies were exposed to one of the following three

338 conditions: Trained-Fed, Trained-Starved and Untrained-Fed. Only Trained-Fed flies are

339 expected to increase sleep after treatment and thus form sleep-dependent memory

340 (Chouhan et al., 2021). Brain dissection, single cell suspension and cell sorting were

341 used to extract ap α′/β′ neurons in each of these three different conditions, and bulk-

342 sequencing of the sorted cells was conducted. (B) We sequenced four samples for each

343 condition and subjected them to differential gene analysis of pairwise comparison of the
344 three conditions. We found that 59 genes are significantly different in TrainedStarved
345 vs. TrainedFed and UntrainedFed vs. TrainedFed. (C) Gene ontology (GO) analysis of
346 these 59 genes using FlyEnrichr (https://maayanlab.cloud/FlyEnrichr/) revealed that
347 they encode cellular components of the 90S preribosome, Cajal body, DNA-directed
348 RNA polymerase complex, nuclear euchromatin, and condensed chromosome.(D)
349 Heatmap of the 59 DEGs including two genes labeled in red, CG13773 (Polr1F) and
350 Regnase-1, which affect sleep and are the focus of this study due to their impacts on
351 sleep. DEGs were identified by DESeq2 with the cutoff of FDR< 0.1 and fold
352 change >1.5. Heatmaps were plotted by using TPM values of genes for each sample;
353 data were log-transformed and scaled row-wise for visualization.

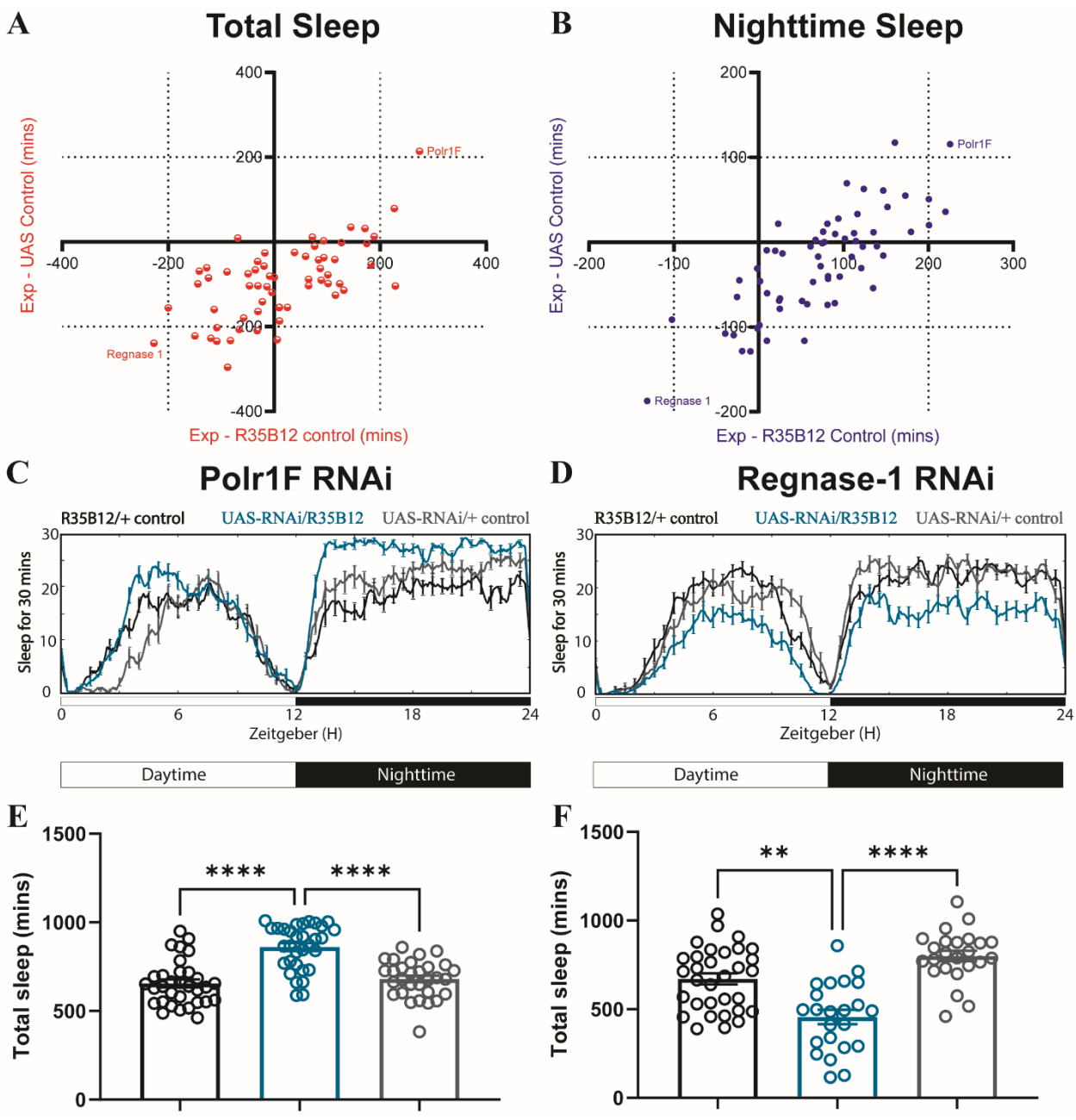



**Figure 2. The sleep screen of differentially expressed genes identifies Polr1F and Regnase-1 as sleep-regulating genes.** (A-B) Flies carrying the ap α′/β′ neuron driver R35B12-Gal4 were crossed with flies carrying UAS-RNAi constructs targeting DEGs identified from RNA-seq analysis. 5-7 days old female F1 progeny were loaded onto Trikinetics DAM monitors to measure their sleep in a 12-hour light: 12-hour dark (12:12

360 LD) cycle. Mean total sleep (A) and nighttime sleep (B) were calculated by Pysolo and
361 the difference between experimental flies and Gal4 and RNAi controls was calculated
362 separately for each independent experiment; average values comparing each
363 experimental to its Gal4 control (X-axis) and RNAi control (Y-axis) are shown in the
364 plots. Of all the lines screened, knockdown of Polr1F and Regnase-1 had strongest
365 effects on sleep, producing an increase and decrease in sleep respectively. (C-F) show
366 the representative sleep traces of *R35B12-Gal4>polr1F*$^{RNAi}$ flies and *R35B12-*
367 *Gal4>regnase1*$^{RNAi}$. N = 23-32 per genotype from two independent replicates combined
368 are shown in E and F respectively, and bar graphs show mean + SEM. p values for
369 each comparison were calculated using the Kruskal-Wallis test with Dunn's multiple
370 comparisons test. **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

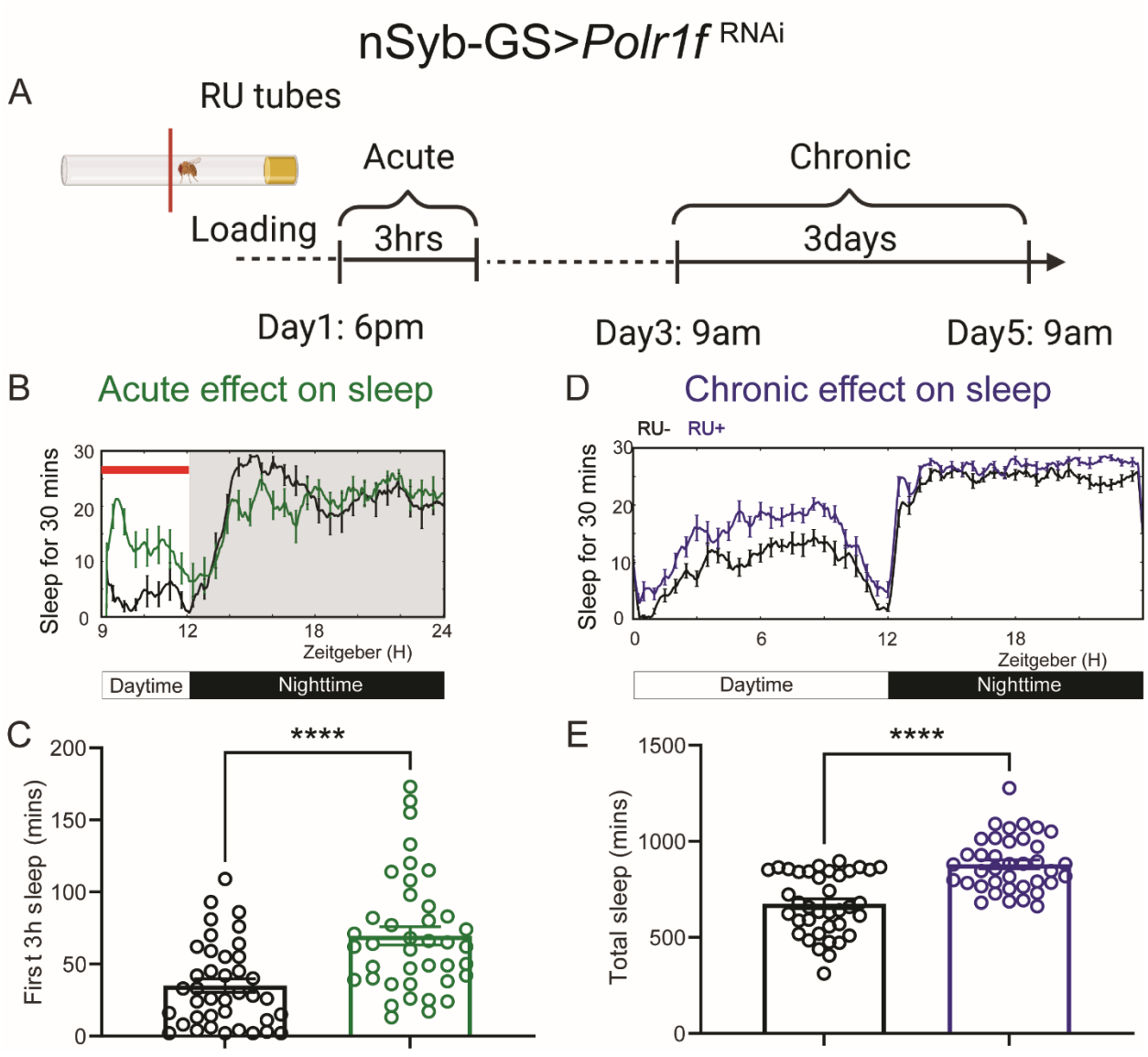
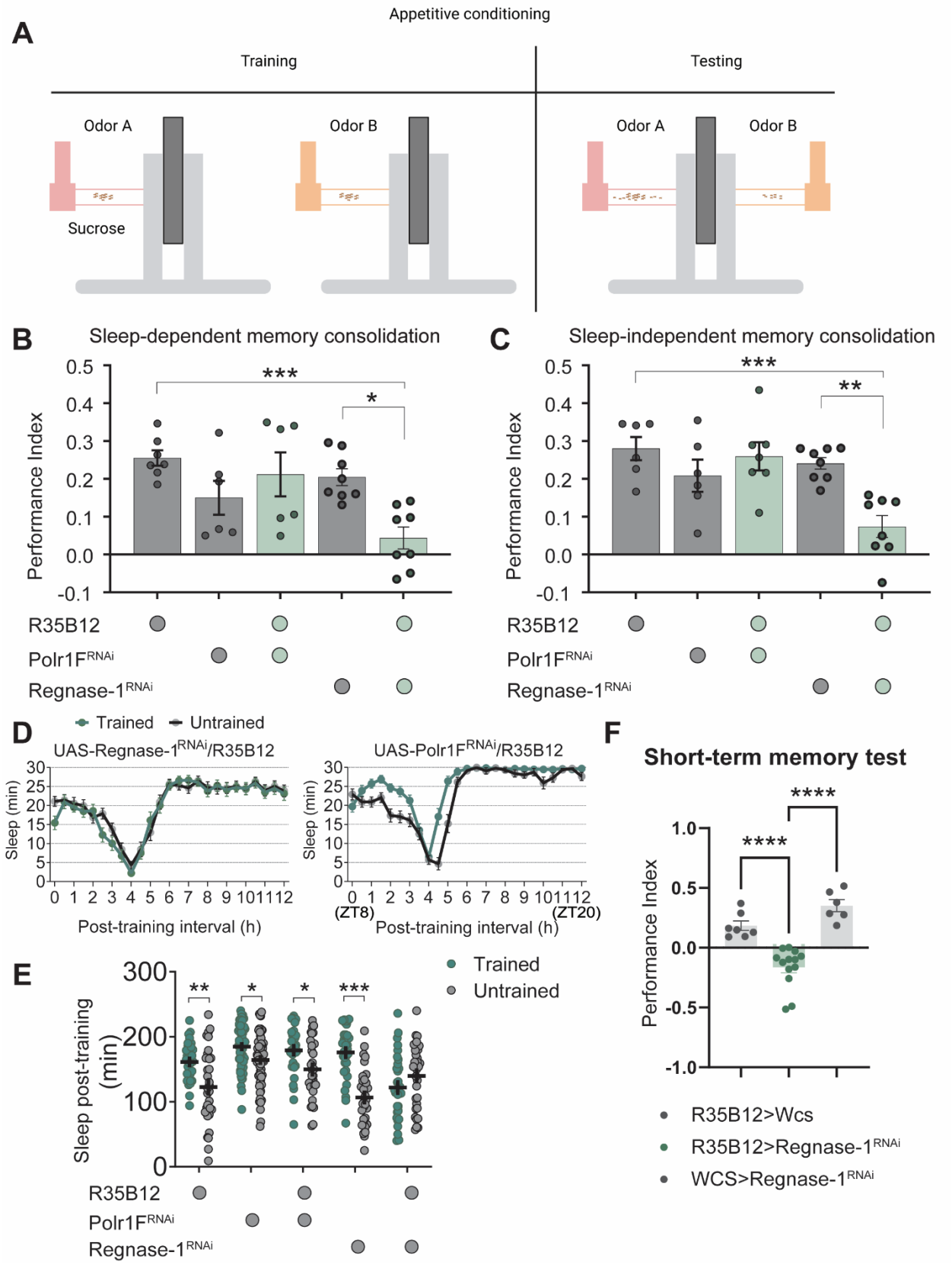



**Figure 3. Acute and chronic effects of pan-neuronal knockdown of Polr1F on sleep in adult flies.** (A) Schematic representation of transient and chronic sleep measurements in *nSyb-GS>polr1f*$^{RNAi}$ flies. (B) Representative sleep traces and transient activity plot of flies expressing Polr1F RNAi under the control of an inducible pan-neuronal driver (*nSyb-GS>polr1f RNAi*) with and without RU treatment. (C) Quantification of sleep during the first 3 hours (ZT 9-12) after F1 progeny flies were loaded into RU- or RU+ DAM tubes at ZT8-T9. Sleep was measured starting at ZT9. N

379 = 39-40 individual flies per replicate with data from three independent replicates
380 combined. The Mann-Whitney test was used to compare RU+ group and RU- groups.
381 (D) Representative average sleep traces of *nSyb-GS>polr1f RNAi* in the RU- and RU+
382 DAM tubes for three consecutive days. Chronic sleep effects of pan-neuronal
383 knockdown Polr1F were measured based on sleep data from day 3 to day 5. (E)
384 Quantification of average total sleep of *nSyb-GS>polr1f*$^{RNAi}$ and controls in the DAM
385 tubes from (D). Unpaired t-test was used to compare between RU- and RU+ groups.
386 ****$p<0.0001$.


A
Appetitive conditioning
Training
Testing
Odor A
Odor B
Sucrose
Odor A
Odor B
B
Sleep-dependent memory consolidation
Performance Index
R35B12
Polr1F^RNAi
Regnase-1^RNAi
C
Sleep-independent memory consolidation
Performance Index
R35B12
Polr1F^RNAi
Regnase-1^RNAi
D
Trained
Untrained
UAS-Regnase-1^RNAi/R35B12
UAS-Polr1F^RNAi/R35B12
Sleep (min)
Post-training interval (h)
(ZT8)
(ZT20)
E
Trained
Untrained
Sleep post-training (min)
R35B12
Polr1F^RNAi
Regnase-1^RNAi
F
Short-term memory test
Performance Index
R35B12>Wcs
R35B12>Regnase-1^RNAi
WCS>Regnase-1^RNAi

387

388 **Figure 4. Regnase-1 expression is essential for sleep-dependent and sleep-**
389 **independent memory.** (A) Schematic representation of the memory test protocol. (B,
390 C) Sleep-dependent and sleep-independent memory tests were conducted under fed
391 and starved conditions, respectively. Knockdown of Regnase-1 significantly reduces
392 long-term memory performance in both fed and starved flies. However, knockdown of
393 Polr1F in ap α′/β′ neurons does not affect long-term memory performance. N≥6
394 biological replicates, each replicate containing 100-150 flies. (D, E) Fed UAS-regnase-
395 1-RNAi/+ and R35B12/+ flies exhibit a significant increase in sleep after training, while
396 *R35B12-Gal4>regnase-1 RNAi* flies fail to show a comparable increase in post-training
397 sleep. The total sleep in the ZT8-ZT12 interval is shown in (E). Polr1F knockdown in ap
398 α′/β′ neurons does not affect the post-training increase in sleep. N≥32. (F) Compared to
399 R35B12-Gal4/+ and +/UAS-Regnase-1 RNAi flies, *R35B12-Gal4>reganse-1 RNAi* flies
400 show a significant decrease in the performance index in short-term memory. N≥6
401 biological replicates, each containing 100-150 flies. ns=not significant, $p>0.05$, *$p<0.05$,
402 **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

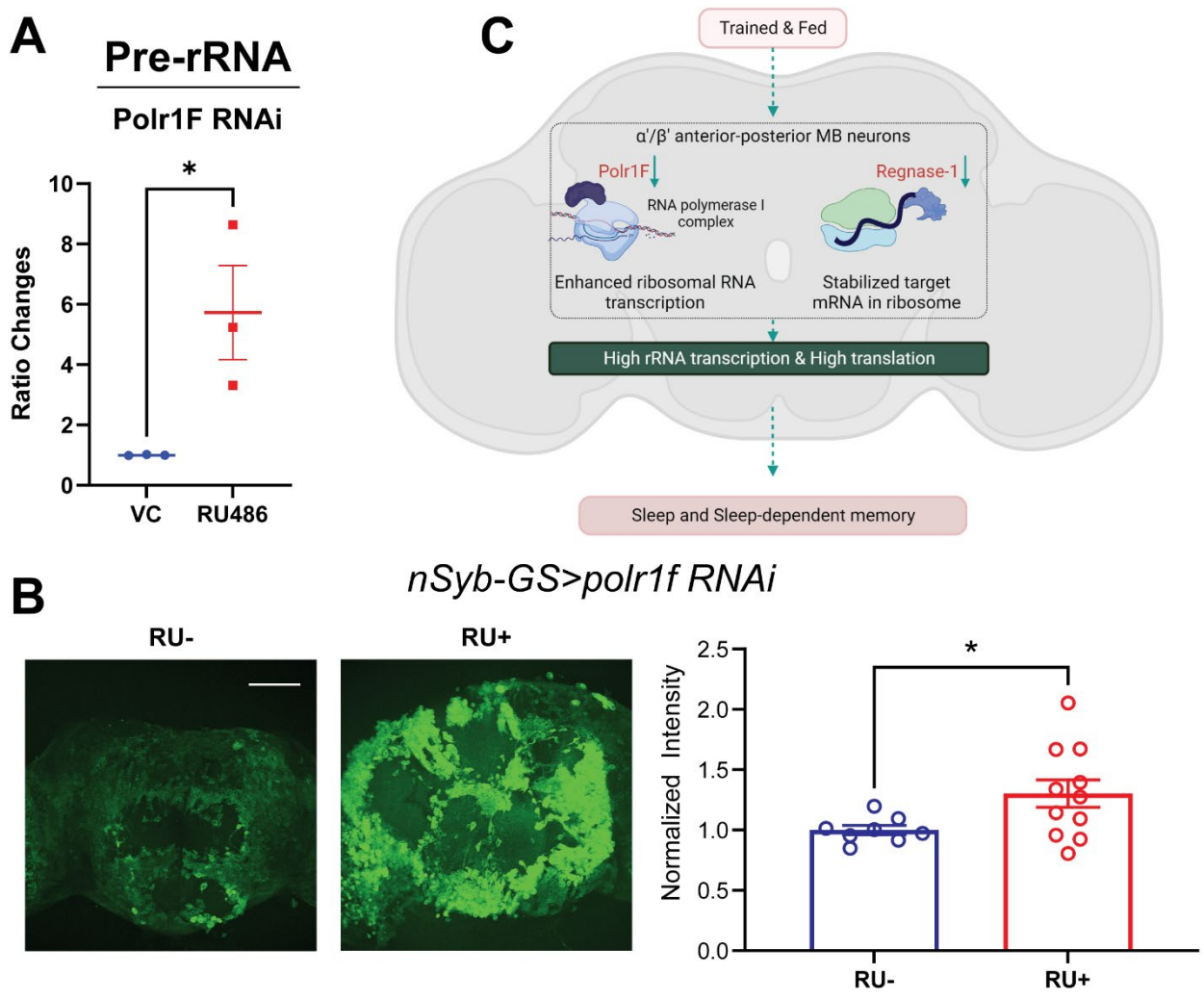



**Figure 5. Knockdown of Polr1F results in high translation.** (A) The *nSyb-GS>polr1f RNAi* flies exhibit a significant increase in pre-rRNA levels. (B) The ex-vivo puromycin immunostaining assay was used to measure translation in dissected whole brains. The results show that knockdown of Polr1F using the pan-neuronal nSyb-GeneSwitch (GS) system increases translation relative to control flies that were not treated with RU. The normalized mean grayscales from the RU- and RU+ groups are compared using an unpaired t-test. The analysis includes data from 8-11 flies per group, with results from two independent replicates combined. Scale bar: 100 μm. (C) The schematic model illustrates the roles of Polr1F and Regnase-1 in memory consolidation. The genes

413 Polr1F and Regnase-1 are prominently downregulated during memory consolidation in
414 trained and fed flies, respectively. Polr1F is involved in regulating ribosomal RNA
415 synthesis, and its decrease in levels in trained and fed flies promotes sleep and
416 translation. In contrast, Regnase-1 is involved in mRNA decay, and its downregulation
417 during memory consolidation may contribute to the stabilization of mRNAs that encode
418 proteins important for long-term memory formation.

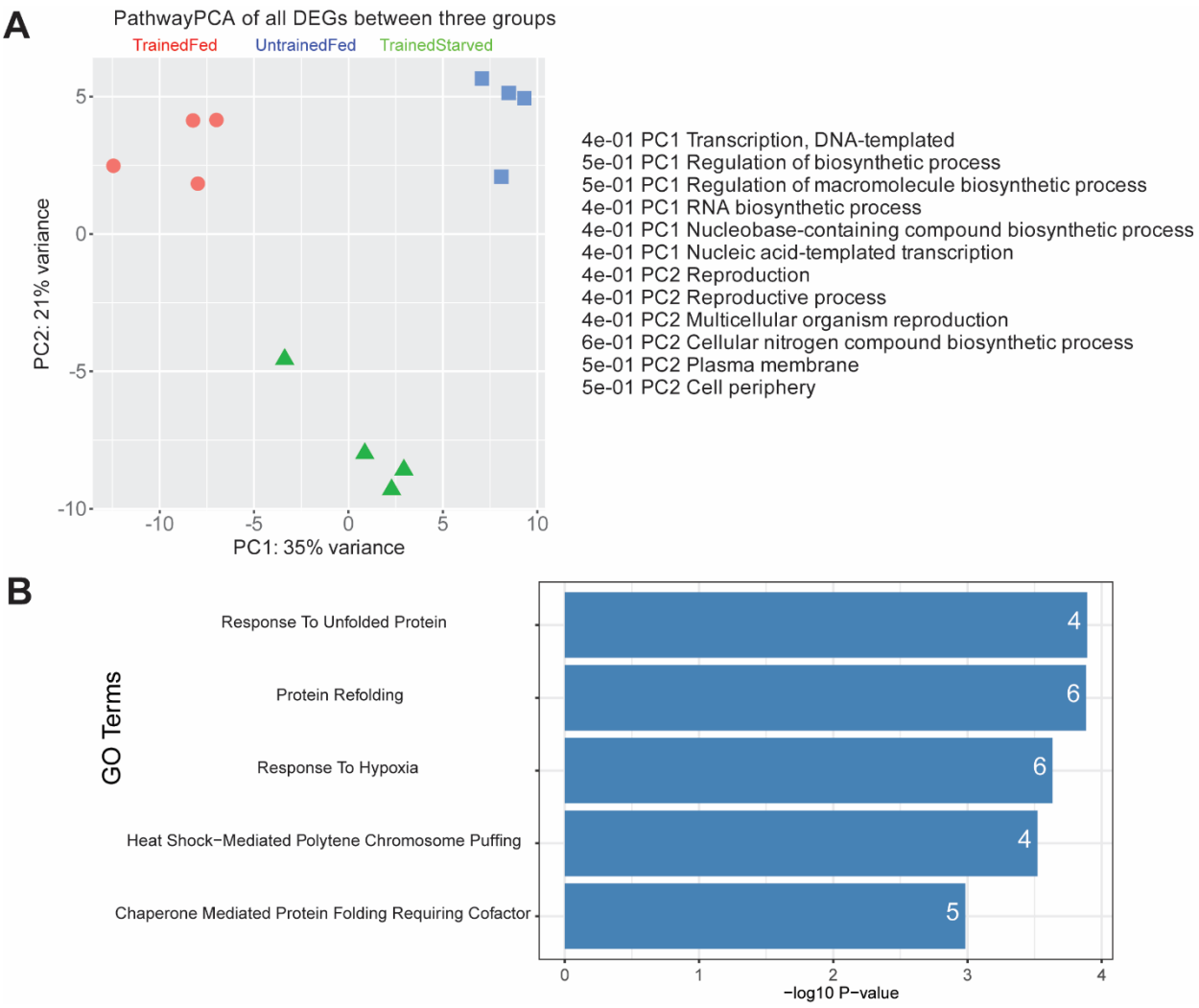



**Figure 1 – supplemental figure 1.** (A) PathwayPCA plot of all the differentially expressed genes (DEGs) across the three different conditions. The plot shows that genes responsible for PC1, which accounted for 35% of variance, largely encode proteins involved in transcription and biosynthesis, including RNA biosynthesis processes. (B) Top5 significant Gene Ontology (GO) biological process terms of all differentially expressed genes (DEGs) from three pairwise comparisons are shown. The x-axis represents the -log10 of the P-value, and the numbers on the bars indicate the count of genes identified for each GO term.

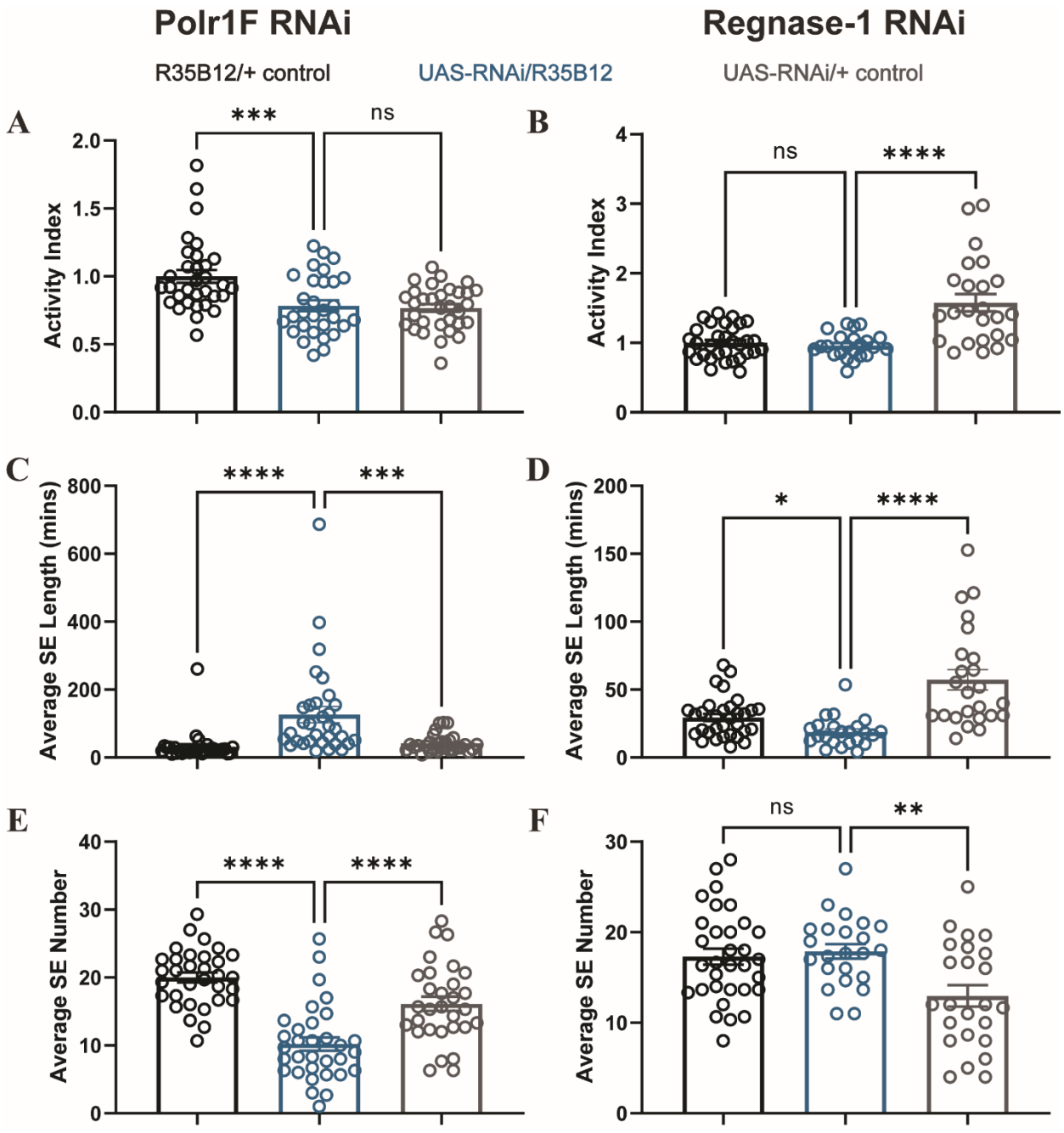



**Figure 2 – supplemental figure 1. Effect of Polr1F and Regnase-1 knockdown on activity and sleep architecture.** (A-B) Total activity of flies is not altered by knockdown of Polr1F and Regnase-1. One-way ANOVA was used to calculate the p-values for each comparison. (C-D) Knockdown of Polr1F significantly increases the nighttime average sleep episode (SE) length in R35B12-Gal4>polr1F RNAi flies, while knockdown of

434 Regnase-1 reduces it in R35B12-Gal4>regnase1 RNAi flies. The p-values for each
435 comparison were calculated using the Kruskal–Wallis test with Dunn′s multiple
436 comparisons test. (E-F) Knockdown of Polr1F significantly reduces the nighttime
437 average sleep episode (SE) number in R35B12-Gal4>polr1FRNAi flies, but it is not
438 significant compared to control groups in R35B12-Gal4>regnase1RNAi flies. The p-
439 values for each comparison were calculated using the Kruskal–Wallis test with Dunn′s
440 multiple comparisons test. ns = not significant, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p <$
441 $0.001$, ****$p < 0.0001$.

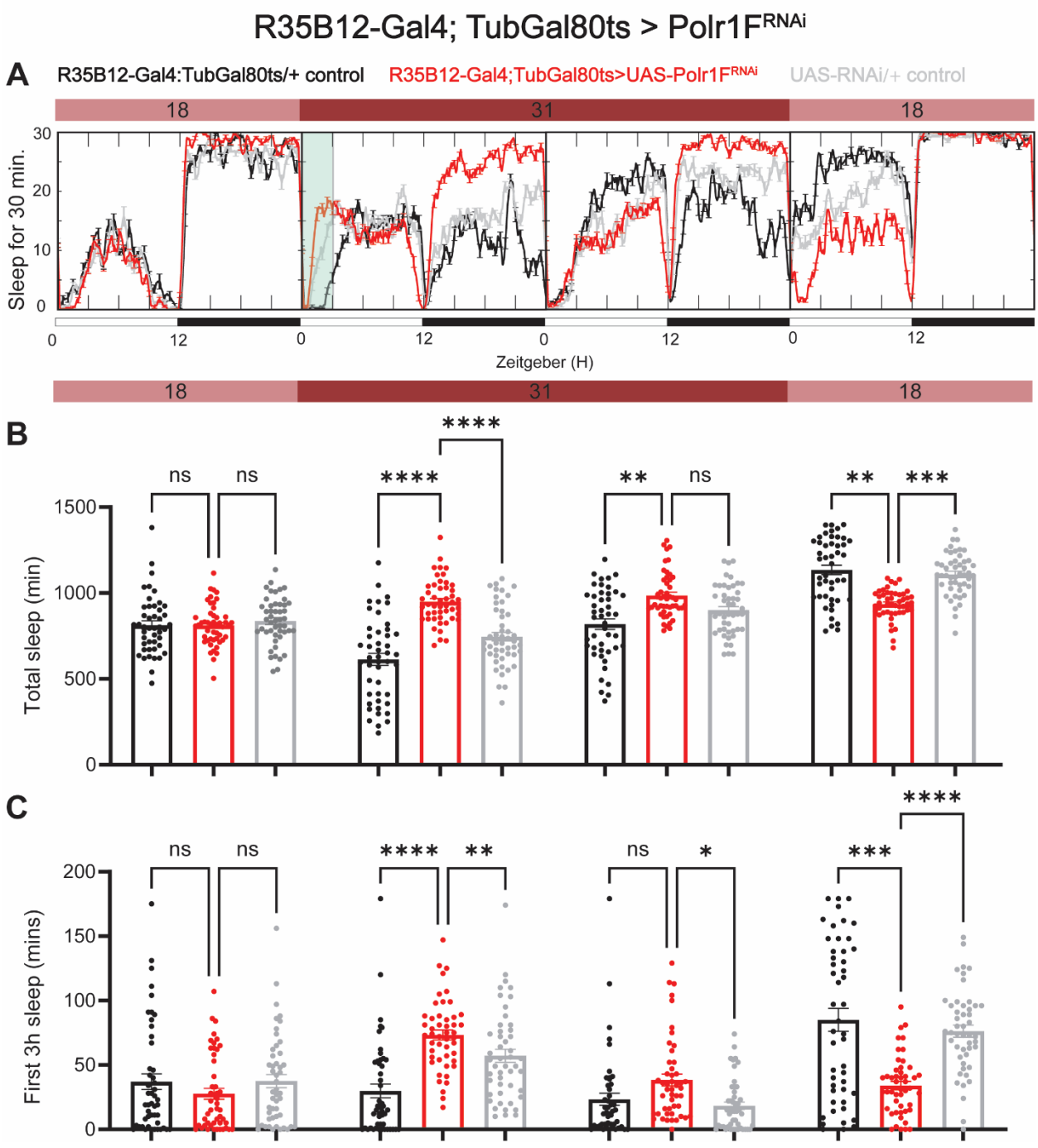



**Figure 3 – supplemental figure 1. Adult specific knockdown of Polr1F promotes sleep.** (A) Representative sleep traces of adult flies expressing Polr1F RNAi under the control of R35B12-Gal4/Tubulin-gal80ts. At permissive temperature, Gal80ts inhibits Gal4 activation of UAS; however, upon switching to restrictive temperature, Gal80ts is

447 deactivated, leading to downstream activation of the UAS-regulated gene. (B) Sleep
448 patterns were quantified over four consecutive days, with flies under permissive
449 temperature on the first and last days and restrictive temperature on the second and
450 third days. (C) Sleep quantification during the initial 3-hour period (ZT 0-3) after light
451 onset for each day is shown. N = 46-48 individual flies per replicate with data from three
452 independent replicates combined. The Kruskal-Wallis test with Dunn′s multiple
453 comparisons test was used to compare three groups. ns = not significant, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

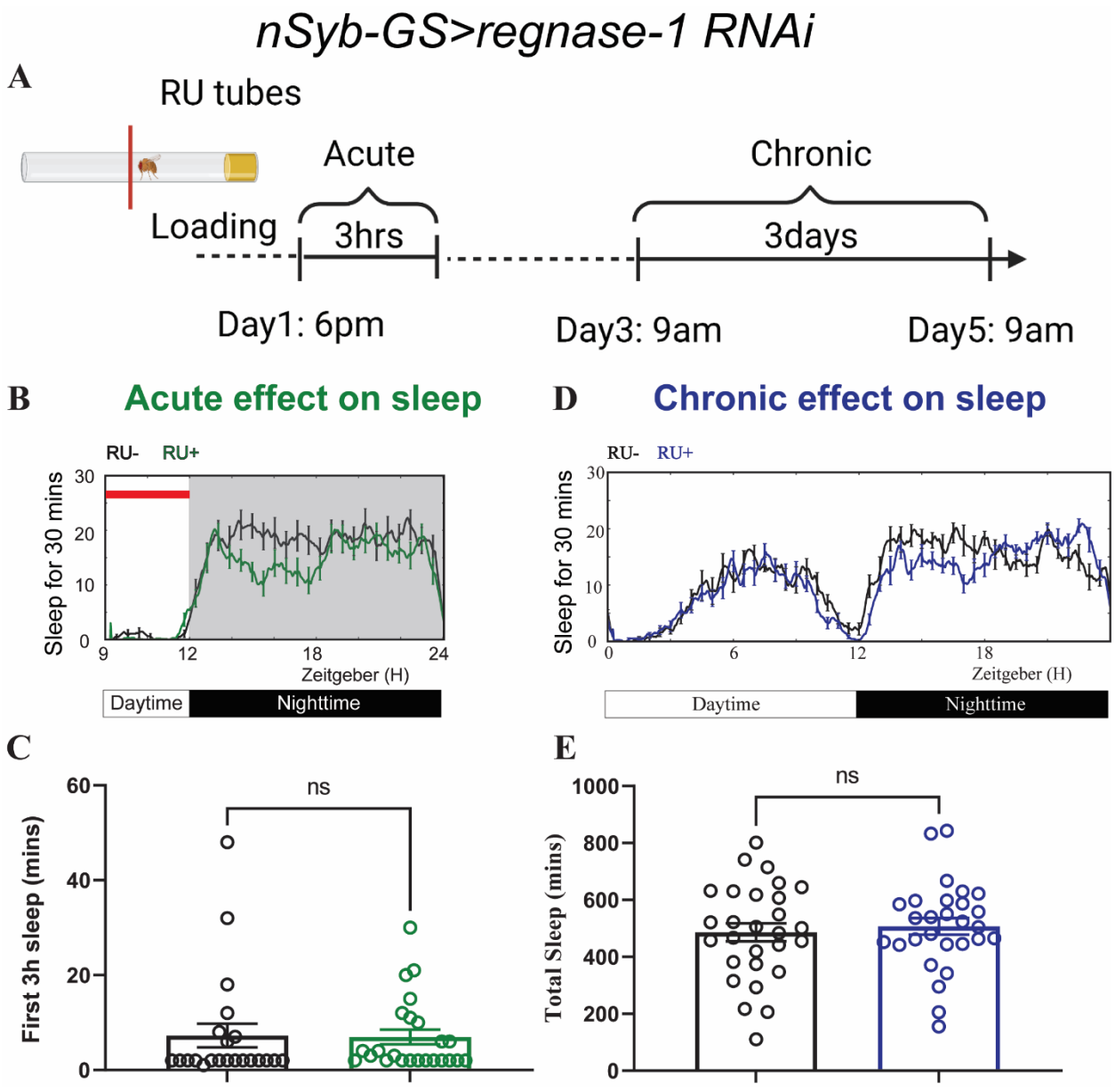
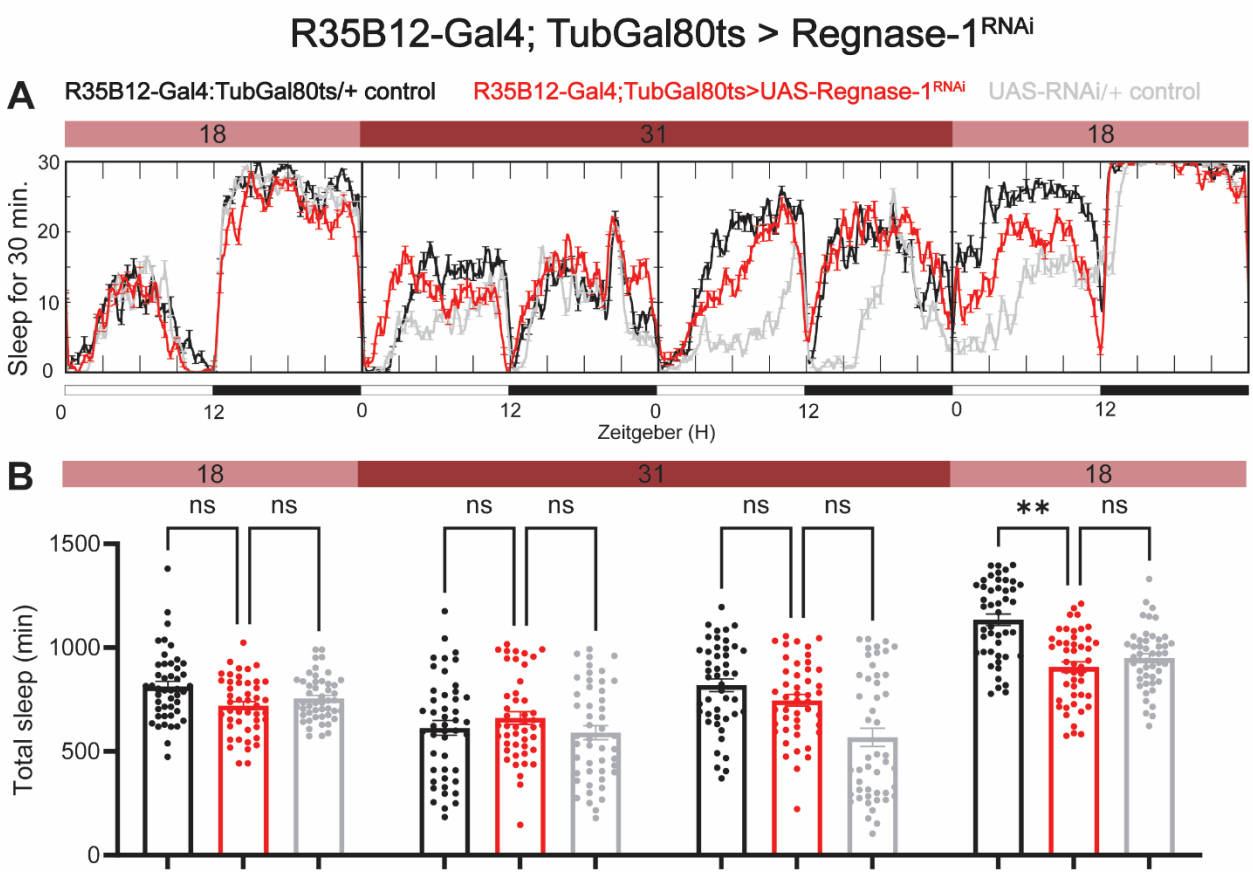



**Figure 3 – supplemental figure 2. Acute and chronic effects of pan-neuronal knockdown of Regnase-1 on sleep in adult flies.** (A) Schematic representation of the protocol for transient and chronic sleep measurements in *nSyb-GS>regnase-1 RNAi* flies. (B) Representative sleep traces plot of flies expressing Rengase-1 RNAi under the control of an inducible pan-neuronal driver (*nSyb-GS>regnase-1 RNAi*) with and without RU treatment (C) Quantification of sleep during the first 3 hours (ZT 9-12) after F1

462 progeny flies were loaded into RU- or RU+ DAM tubes at ZT8-T9. Sleep was measured
463 starting at ZT9. N = 22-24 individual flies per replicate with data from two independent
464 replicates combined. The Mann-Whitney test was used to compare RU+ group and RU-
465 groups. (D) Representative average sleep traces of *nSyb-GS>regnase-1 RNAi* in the
466 RU- and RU+ DAM tubes for three consecutive days. Chronic sleep effects of
467 knockdown of Regnase-1 were measured based on sleep data from day 3 to day 5. (E)
468 Quantification of average total sleep of *nSyb-GS>regnase-1 RNAi* and controls in the
469 DAM tubes from (D). Unpaired t-test was used to compare between RU- and RU+
470 group. ns = not significant.




471

472 **Figure 3 – supplemental figure 3. Adult specific knockdown of Regnase-1 has no**

473 **effect on sleep.** (A) Representative sleep traces of adult flies expressing Regnase-1

474 RNAi under the control of R35B12-Gal4/Tubulin-gal80ts. (B) Sleep was quantified over

475 four consecutive days, with flies under permissive temperature on the first and last days

476 and restrictive temperature on the second and third days. N = 46 individual flies per

477 replicate with data from three independent replicates combined. The Kruskal-Wallis test

478 with Dunn's multiple comparisons test was used to compare three groups. ns = not

479 significant, $p > 0.05$, $*p < 0.05$, $**p < 0.01$.

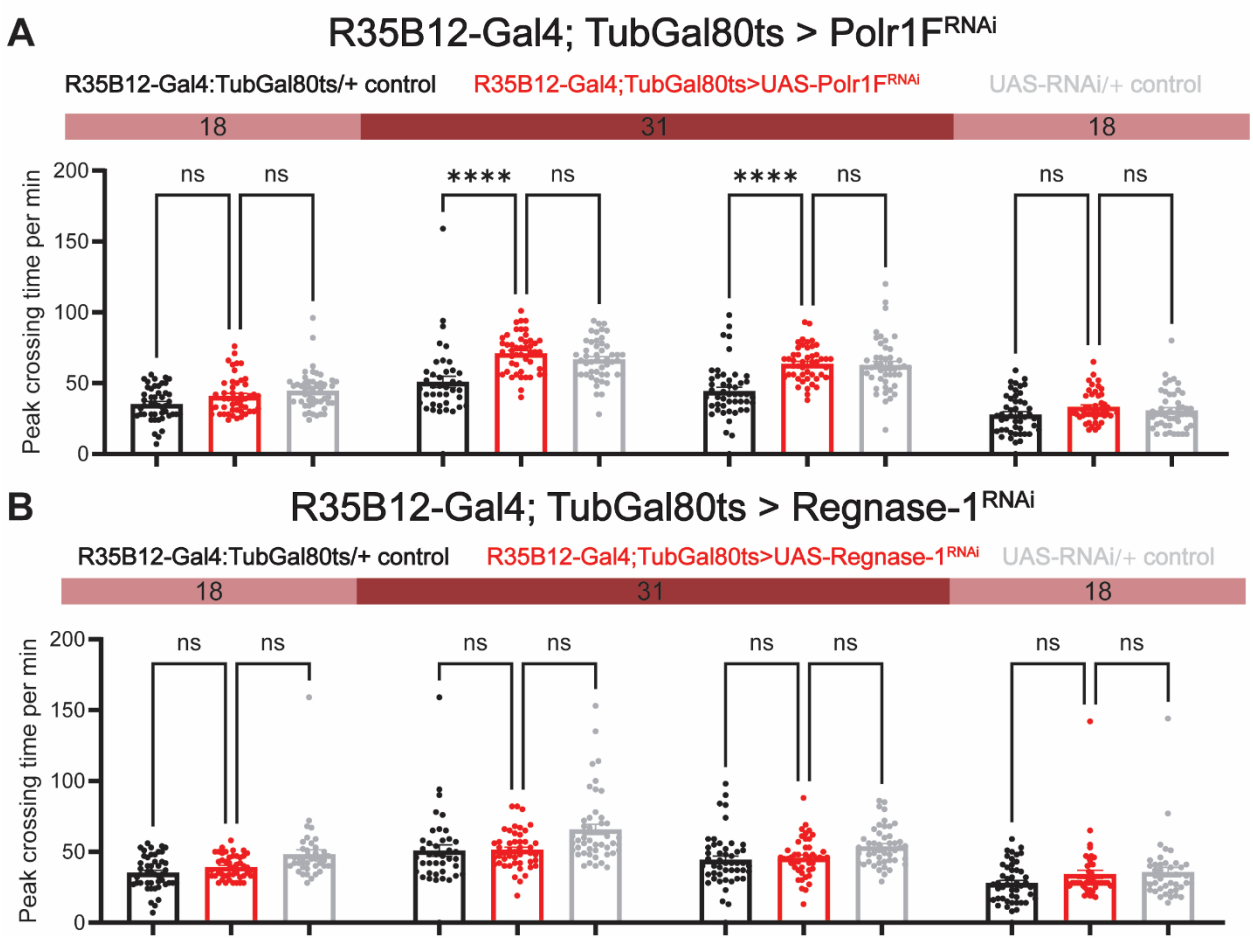
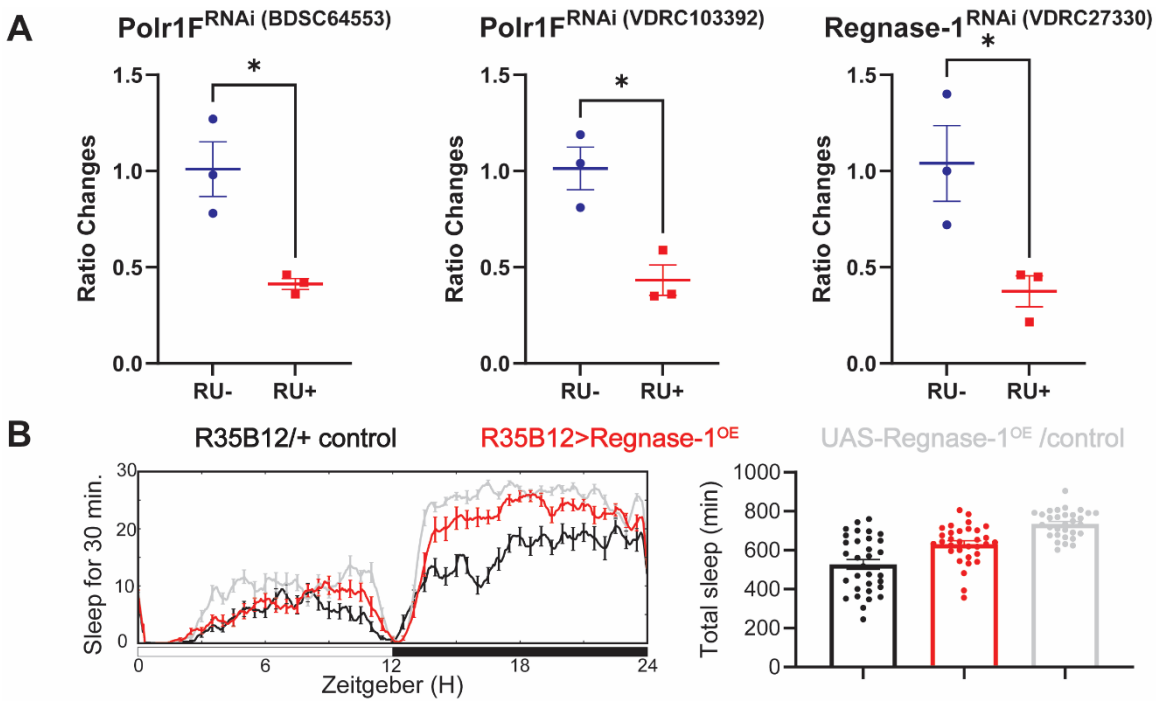



**Figure 3 – supplemental figure 4. Adult specific knockdown of Polr1F or Regnase-1 has no effect on peak activity.** (A) Maximal activity per minute for R35B12-Gal4; TubGal80ts>polr1F RNAi was quantified over four consecutive days, with flies under permissive temperature on the first and last days and restrictive temperature on the second and third days. (B) Maximal activity per minute for R35B12-Gal4; TubGal80ts>Regnase-1 RNAi was quantified. N = 40-48 individual flies per replicate with data from three independent replicates combined. The Kruskal-Wallis test with Dunn's multiple comparisons test was used to compare three groups. ns = not significant, $p > 0.05$, *****$p < 0.0001$.




490

491 **Figure 4 – supplemental figure 1. Regnase-1 overexpression in the ap α′/β′**

492 **neurons does not affect sleep.** (A) The efficacy of the RNAi used in the experiments

493 was verified by real-time quantitative PCR. The different RNAi lines were crossed with

494 *nSyb-GS*, and adult progeny were placed in either RU- or RU+ tubes overnight before

495 being dissected for qPCR. Welch's test was used for statistical analysis across all three

496 RNAi, with *p<0.05 indicating significance. (B) Representative sleep traces and

497 quantification of adult flies expressing Regnase-1 under the control of R35B12-Gal4.

498 Sleep data were averaged over three consecutive days, with N = 31-32 individual flies

499 per replicate, and combined data from two independent replicates were analyzed.

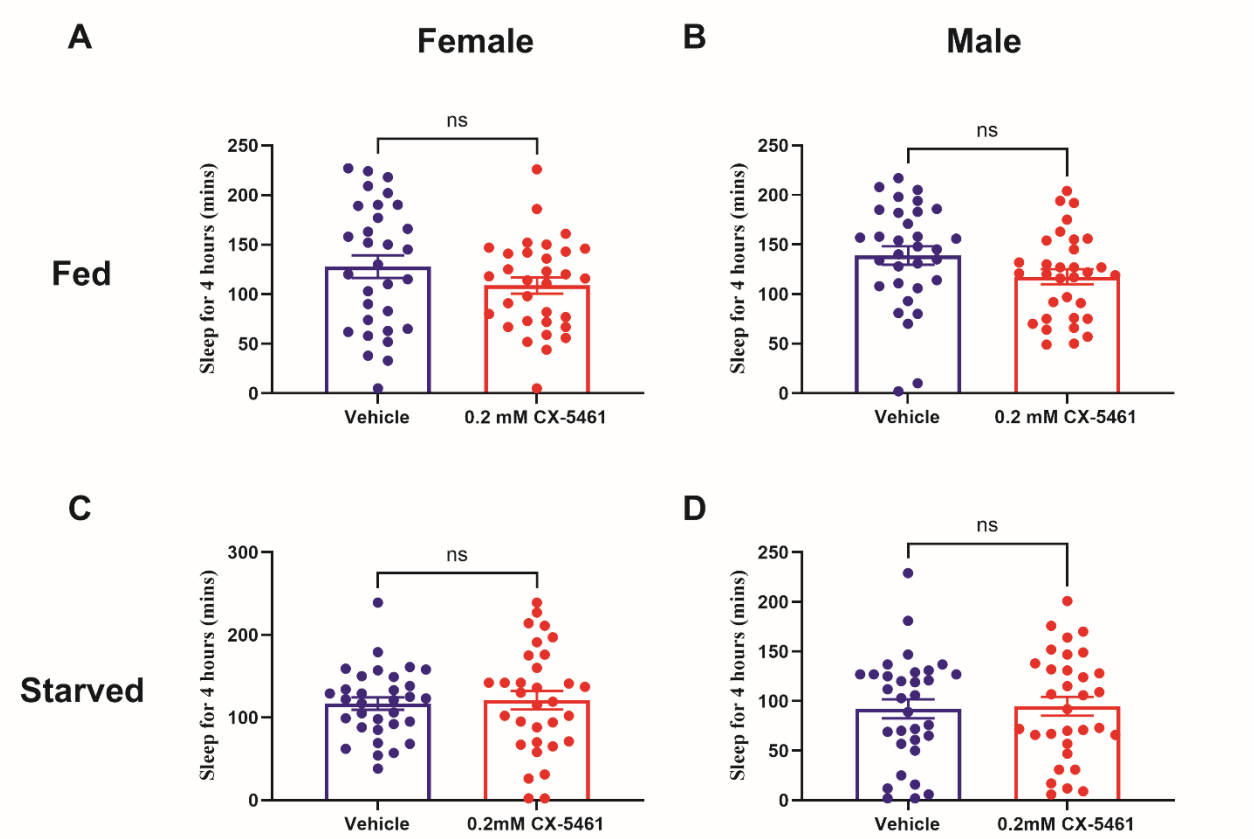



**Figure 5 – supplemental figure 1. rRNA inhibitor (CX-5461) feeding does not affect sleep.** Feeding of the ribosome RNA inhibitor CX-5461 at a concentration of 0.2mM does not affect sleep in fed or starved flies. Statistical analysis shows that there is no significant difference between CX-5461-fed and control flies. The sample size is N=32 per group from two independent replicates. ns, not significant).

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632

633 **Conflict of interest statement**

634 The authors declare no competing interests.

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641 line.

642 **Author contributions**

643 Conceptualization, Y.J.L., N.S.C. and A.S.; Methodology, Y.J.L., N.S.C., S.Z., S.B.N.
644 and R.M. Software, Y.J.L; Validation, Y.J.L., N.S.C., S.Z., S.B.N., R.M., J.S., A.K. and
645 Z.F.Y.; Formal analysis, Y.J.L., N.S.C. and R.M.; Investigation, Y.J.L. and N.S.C.;
646 Resources, Y.J.L., N.S.C., S.Z., R.M., J.S., A.K. and Z.F.Y.; Data Curation, Y.J.L.,
647 N.S.C. and R.M.; Writing – Original Draft, Y.J.L. and N.S.C. Writing – Review & Editing,
648 Y.J.L., N.S.C. and A.S. Visualization, Y.J.L., N.S.C. and R.M.; Supervision, Y.J.L.,
649 N.S.C. and A.S.; Project administration, A.S.; Funding acquisition, A.S.ss

650 **Materials and Methods**

651 **Contact for reagent and resource sharing.**

652 Amita Sehgal (amita@pennmedicine.upenn.edu)

653

654 **Key resource table**

655 See Appendix 1.

656

657 **Fly stock and maintenance**

658 All the stock information of the flies used in this project are listed in the key resource
659 table and flies were reared on the standard cornmeal vials or bottles at 25 °C with 12:12
660 hours light dark cycle in the preset incubator. The genetic background control used in
661 the paper is White-CantonS (wCS) unless specified.

662

663 **Behavior measurement in *Drosophila***

664 We have used both single beam and updated multibeam *Drosophila* activity monitoring
665 (DAM) system from Trikinetics (https://trikinetics.com/) in our experiments. Briefly, 5-7
666 days old female flies were loaded into 60/90 mm glass locomotor tubes for behavior
667 tests, using DAM2/5H *Drosophila* activity monitors from Trikinetics. 1/15 infrared beams
668 bisect each tube, providing movement (position in multibeam) information of the fly
669 across the tube. Locomotor tubes are loaded with 2% agar with 5% sucrose as fly food
670 on one side, and yarn is put on the other side to restrain the behavior of flies inside the
671 glass tubes. For experiments with the inducible Gene Switch system, 0.5mM RU486
672 (mifepristone) was added to the fly food to activate the expression of the transgenes

673 under the control of UAS. Three constitutive days of data were used for sleep analysis
674 by Pysolo (https://www.pysolo.net/).
675
676 **Appetitive conditioning**
677 ~100 4-7 day old mixed-sexes flies were starved for 12 hours in *Drosophila* bottles with
678 water -soaked filtered paper and then trained at 25°C and 70% relative humidity to
679 associate sucrose with odor A for 2 minutes, and then a blank with odor B for 2 minutes
680 with 30-second clean air in between. The odors used were 4-Methylcyclohexanol (MCH)
681 and 3-Octanol (OCT) in paraffin oil and the concentration for both MCH and OCT in oil
682 was 1:10. After conditioning, flies were moved back to normal fly food or starved for 1
683 hour and dissected for subsequent ap cell sorting and RNA-sequencing. For the short-
684 term memory test, flies were tested immediately after conditioning in the same wheel for
685 2 mins.
686
687 To assess post-training sleep, flies were introduced in glass tubes containing 2% agar
688 and 5% sucrose through an aspirator without anesthesia and loaded into the DAM
689 system after training. For long-term memory assessment, trained flies were either kept
690 on food vials for 24 h or were further starved. Starved flies were tested for memory 24 h
691 after training, while fed flies were re-starved for 42 h before memory tests. Memory was
692 tested by giving flies a choice between odor A and odor B for 2 min in a T-maze.
693 Performance index (PI) was calculated as the number of flies selecting $CS^+$ odor minus
694 the number of flies selecting $CS^-$ odor divided by the total number of flies. Each PI is the

695 average of PIs from reciprocal experiments with two odors swapped to minimize non-
696 associative effects.
697
698 **Cell isolation and sorting**
699 Dissected brains are dissociated by following the protocol from (Li et al., 2017). Briefly,
700 brains are dissected in Schneider′s medium, and then are placed in a shaker and
701 dissociated in Papain solution, filtered through a 100 $\mu$m cell strainer, and re-suspended
702 in Schneider′s medium. 500 GFP+ cells from the same conditions were sorted into 96
703 well microplate with lysis buffer from Smart-seq2 HT kit and frozen. We dissected 50
704 brains for each group to ensure enough GFP+ cells. Cell sorting were conducted by
705 either BD FACSMelody or BD FACSAria (BD Biosciences), and dead cells were
706 excluded with 4′, 6-diamidino-2-phenylindole (DAPI). Doublets were excluded using and
707 forward scatter (FSC-H by FSC-W) and side scatter (SSC-H by SSC-W). Size of cells
708 was selected by FSC-A by FSC-A and validated for fly neurons using cells from flies
709 expressing nsyb-nGFP. Length of time from tissue harvest to cell collection
710 approximated 4 hours.
711
712 **RNA-seq and data analysis**
713 GFP+ cells were sorted and immediately frozen, then sent to Admera Health
714 (https://www.admerahealth.com/) for RNA extraction, RNA library construction, and
715 sequencing using the Smart-seq2 HT kit. To analyze the RNA-sequence data, we used
716 Hisat2 (http://daehwankimlab.github.io/hisat2/) to map the sequencing data FASTQ files
717 to the fly genome (BDSG6). The alignment results were then counted by LiBiNorm

718 (https://warwick.ac.uk/fac/sci/lifesci/research/libinorm/) using the GENCODE reference
719 genome. Raw count and TPM were used separately in further analysis. Raw count data
720 were analyzed by IDEP v0.95 to identify genes expressed differentially between three
721 conditions (Ge et al., 2018). We filtered out low-expressed genes using a cutoff of
722 CPM>0.5, at least detected in 3 independent samples, and treated missing values as
723 gene median. Regularized log transformation was used to transform raw count data for
724 clustering and PCA. Differentially expressed genes were identified using DESeq2 with
725 an FDR cutoff of 0.1 and minimum fold change of 2.
726

727 **Puromycin assay and imaging**

728 We developed a puromycin assay to measure the rate and localization of nascent
729 peptide synthesis in the fly brain, and similar method has been described in the fly
730 larvae (Deliu et al., 2017). Fly brains were dissected in Schneiders′ medium and
731 incubated with puromycin for 40 min *in vitro* to allow puromycin to incorporate into the
732 newly synthesized peptide. Subsequently, using an anti-puromycin antibody, standard
733 immunostaining protocols were applied to detect the number and position of newly
734 synthesized puromycin-tagged peptides (Aviner, 2020), which provided an estimate of
735 translation rate. The brains were then imaged with a 20X oil immersion confocal
736 microscope with a resolution of 1024*1024. The GFP intensity of the images was then
737 measured by Fiji software (ImageJ) and the average intensity of the samples was
738 analyzed for comparison.
739

740 **RNA polymerase I inhibitor II, CX-5461 protocol**

741 CX-5461 was mixed with 2% agar and 5% sucrose to make fly motor tubes at a final
742 concentration of 0.2 mM. Flies are loaded into these CX-5461 tubes or vehicle control
743 tubes 4 hours before light turns off and transient sleep changes are measured.
744

745 **Quantitative Real-time PCR (qPCR)**

746 10 flies′ brains from each group were dissected for RNA extraction using Qiagen's
747 RNeasy Plus Mini Kit. Total RNA was then reverse transcribed to cDNA by using
748 random hexamers and Superscript II from Invitrogen. qPCR was performed using
749 SYBR-green master mix and oligonucleotide information is provided in the Key
750 Resources table. Relative gene expression was analyzed using the ΔΔCt method.
751

752 **Statistical Analysis**

753 Fly sleep behavioral data extracted from Pysolo was analyzed by GraphPad Prism
754 (https://www.graphpad.com/). Data from different replicates were pooled directly and
755 first tested for normality using D'Agostino-Pearson and Shapiro-Wilk tests. For normally
756 distributed data, unpaired parametric Student's t-test is used for two-sample
757 experiments and one-way ANOVA with Turkey post hoc test for three-sample or more
758 experiments. Non-normally distributed data were analyzed using nonparametric tests,
759 the Mann-Whitney test for two-sample experiments and the Kruskal-Wallis test with
760 Dunn's multiple comparisons test for three or more samples experiments. For all
761 graphs, unless otherwise stated, data are presented as mean and standard error of the
762 mean (SEM) and statistical significance was accepted for $p$ value $< 0.05$.
763

764 **Data availability**

765 Sequencing data is available at NCBI BioProject with accession number

766 PRJNA1132369. All other data for this study are included in the manuscript and

767 supporting files.

768

769 **Supplemental information**

770 Supplemental information includes 8 figures and 1 table.