Title: Modulation of RNA processing genes during sleep-dependent memory

Yongjun Li[1, 2, *], Nitin S. Chouhan[1, 4 *], Shirley Zhang[1, 5], Rebecca S. Moore[1], Sara B. Noya[1], Joy Shon[1], Zhifeng Yue[1], Amita Sehgal[1]

[1]Howard Hughes Medical Institute and Chronobiology and Sleep Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, United States

[2]Department of Biology, University of Pennsylvania, PA 19104, United States

[3]Department of Pharmacology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, United States

[4]Current address: Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400005, India

[5]Current address: Department of Cell Biology, Emory University School of Medicine, Atlanta GA 3032

*Equal contribution

Correspondence should be addressed to Amita Sehgal at amita@pennmedicine.upenn.edu

14 **Keywords**

15 Sleep, memory consolidation, translation, ribosomal RNA, mushroom body

16

17 **Abstract**

18 Memory consolidation in *Drosophila* can be sleep-dependent or sleep-independent,
19 depending on the availability of food. The anterior posterior (ap) alpha′/beta′ (α′/β′)
20 neurons of the mushroom body (MB) are required for sleep-dependent memory
21 consolidation in flies fed after training. These neurons are also involved in the increase
22 of sleep after training, suggesting a coupling of sleep and memory. To better
23 understand the mechanisms underlying sleep and memory consolidation initiation, we
24 analyzed the transcriptome of ap α′/β′ neurons one hour after appetitive memory
25 conditioning. A small number of genes, enriched in RNA processing functions, were
26 differentially expressed in flies fed after training relative to trained and starved flies or
27 untrained flies. Knockdown of each of these differentially expressed genes in the ap
28 α′/β′ neurons revealed notable sleep phenotypes for Polr1F and Regnase-1, both of
29 which decrease in expression after conditioning. Knockdown of Polr1F, a regulator of
30 ribosome RNA transcription, in adult flies promotes sleep and increases pre-ribosome
31 RNA expression as well as overall translation, supporting a function for Polr1F
32 downregulation in sleep-dependent memory. Conversely, while constitutive knockdown
33 of Regnase-1, an mRNA decay protein localized to the ribosome, reduces sleep, adult
34 specific knockdown suggests that effects of Regnase-1 on sleep are developmental in
35 nature. We further tested the role of each gene in memory consolidation. Knockdown of
36 Polr1F does not affect memory, which may be expected from its downregulation during

memory consolidation. Regnase-1 knockdown in ap α′/β′ neurons impairs all memory, including short-term, implicating Regnase-1 in memory, but leaving open the question of why it is downregulated during sleep-dependent memory. Overall, our findings demonstrate that the expression of RNA processing genes is modulated during sleep-dependent memory and, in the case of Polr1F, this modulation likely contributes to increased sleep.

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

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

Since both post-training sleep and memory consolidation occur rapidly within a few hours after training, we investigated transient gene expression changes in the ap α′/β′ neurons after training to elucidate the interplay of sleep and memory consolidation. Here, we profiled the transcriptome of ap α′/β′ neurons 1 hour after flies were fed and trained, as well as under control conditions in which flies were starved and trained or fed and untrained. We knocked down the differentially expressed genes in trained and fed flies and identified two RNA processing genes that affect sleep. Knockdown of one of these, Polr1F, a regulator of ribosomal RNA synthesis, promotes sleep and translation, both of which are required for consolidation of memory in trained and fed flies. Knockdown of Polr1F does not affect memory, which is consistent with downregulation of this gene during memory consolidation. Knockdown of Regnase-1, an mRNA decay protein, reduces sleep and disrupts post-training sleep, and also impairs both short- and long-term memory. While the relevance of Regnase-1 downregulation during sleep-dependent memory is unclear, overall we propose that RNA processing is modulated during sleep-dependent memory, likely to regulate sleep and 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

100 (DEGs), a statistical method to identify key patterns in gene expression data by
101 reducing data dimensionality, showed that genes contributing to PC1, which accounted
102 for 35% of the variance, largely encode proteins involved in transcription and
103 biosynthesis, including RNA biosynthesis processes (**Figure 1 – supplemental figure**
104 **1A**) (Odom et al., 2020). This suggests that the training and feeding paradigm
105 influenced transcription and RNA biosynthetic processes. Meanwhile, gene ontology
106 analysis of these DEGs, performed using DAVID Bioinformatics
107 (https://david.ncifcrf.gov/home.jsp), revealed significant enrichment in processes such
108 as protein unfolding/refolding and the stress response (**Figure 1 – supplemental figure**
109 **1B**) (Sherman et al., 2022).

110 Of all the DEGs across the three groups, we focused on 59 DEGs that were
111 significantly different in TrainedStarved vs. TrainedFed and UntrainedFed vs.
112 TrainedFed; of these, 56 were downregulated and only three were upregulated in the
113 Trained-Fed condition compared to the control conditions (**Figure 1B, D, Figure 1 –**
114 **source data**). Gene ontology (GO) analysis of these 59 genes using FlyEnrichr
115 (https://maayanlab.cloud/FlyEnrichr/) indicated that they encode cellular components of
116 the 90S preribosome, Cajal body, DNA-directed RNA polymerase complex, nuclear
117 euchromatin, and condensed chromosome, consistent with the PCA enrichment (**Figure**
118 **1C**) (Chen et al., 2013). Our transcriptome results suggest that ribosome biosynthesis
119 and transcription are the initial changes in ap α′/β′ neurons of trained and fed flies.

120

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

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

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

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

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

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

175 **Knockdown of Regnase-1 affects memory and post-training sleep**

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

192 On the other hand, constitutive Regnase-1 knockdown in ap α′/β′ neurons
193 resulted in a significant decrease in long-term memory performance in both fed and
194 starved flies and eliminated the increase in post-training sleep (**Figure 4B-E**). These
195 findings suggested that Regnase-1 expression in ap α′/β′ neurons is necessary for both
196 sleep-dependent and sleep-independent memory consolidation. It is also possible that
197 disruption of Regnase-1 in ap α′/β′ neurons affects learning, short-term memory
198 formation, or appetitive memory retrieval (Aso et al., 2014; Shyu et al., 2019; Rutherford
199 et al., 2023). Indeed, short-term memory tests confirmed that Regnase-1 knockdown
200 flies performed significantly worse than control flies (**Figure 4F**). Given that the sleep
201 effect of Regnase-1 is developmental, it is possible that this is also the case for the
202 memory phenotype.

203 Regnase-1 overexpression had no effect on sleep (**Figure 4 -supplemental**
204 **figure 1B**) (Zhu et al., 2019), but knockdown by R26E01-Gal4 in the m α′/β′ neurons
205 also reduced sleep (data not shown), suggesting that Regnase-1 has a broader role that
206 is not restricted to sleep-dependent memory.

207

208 **Knockdown of Polr1F promotes translation**

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

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

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

236 in fed or starved flies (**Figure 5 – supplemental figure 1**), but this negative result
237 cannot be considered conclusive.

238 Using puromycin to address effects of Regnase-1 knockdown on translation
239 revealed an insignificant slight increase in translation (data not shown); given that
240 Regnase-1 may specifically affect pre-existing translationally active mRNA or may act
241 only on specific target mRNAs, its effects on de novo translation may not be obvious.

242 **Discussion**

243 The anterior-posterior (ap) α′/β′ neurons of the mushroom body make critical and
244 privileged contributions to sleep-dependent memory consolidation and post-training
245 sleep (Krashes et al., 2007; Chouhan et al., 2021), but the mechanisms for sleep-
246 dependent memory in these neurons are not known. To address this gap, we conducted
247 transcriptomic analysis of ap α′/β′ neurons from trained and fed flies to identify genes
248 that change rapidly under conditions that drive sleep-dependent memory. Our
249 transcriptome profiling of ap α′/β′ neurons suggests that genes regulating rRNA
250 transcription and translation are altered in the context of sleep-dependent memory
251 consolidation.
252

253 **RNA processing genes mediate sleep-dependent memory consolidation and**
254 **sleep.**

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

264 Kandel, 2015). More importantly, our findings here indicate that alterations of some of
265 RNA processing genes impact sleep as well.
266
267 **Polr1F regulates ribosome RNA synthesis and sleep**
268 Learning-induced changes in gene expression in memory-related neurons are
269 often critical for long-term memory formation (Cavallaro et al., 2002; Hoedjes et al.,
270 2015; Tadi et al., 2015). Our findings with Polr1F implicate changes in Pol I transcription
271 during sleep-dependent memory. Polr1F(Rpa43) is predicted to be part of the RNA
272 polymerase I complex and is involved in DNA-dependent RNA polymerase
273 activity/rDNA transcription in yeast, especially for the initiation of ribosomal RNA
274 (Beckouët et al., 2011; Marygold et al., 2020). As suggested by our data, Polr1F has an
275 inhibitory role in the RNA polymerase I complex, which is also consistent with the
276 aforementioned study that found a 22 amino acid peptide within Polr1F can inhibit rDNA
277 transcription (Rothblum et al., 2014). Based upon our finding that inducible knockdown
278 of Polr1F rapidly promotes translation, it is likely that the rapid and dramatic decline of
279 Polr1F after training in fed flies serves to increase *de novo* ribosome RNA synthesis.
280 This supports the report that ribosomal RNA is induced by learning and required for
281 memory consolidation in mice (Allen et al., 2018). While we do not know how
282 knockdown of Polr1F promotes sleep, an attractive possibility is that higher translation is
283 a result of elevated sleep. Sleep is thought to promote translation and its role in memory
284 consolidation in fed flies could be related to its effect on translation (Seibt and Frank,
285 2012; Chouhan et al., 2021). Alternatively, increased translation or rRNA synthesis
286 could promote sleep. However, translation is typically thought of as a consequence of

287 sleep rather than a cause (Zimmerman et al., 2006). Also, rRNA transcription rates
288 remain constant throughout the day in the liver (Sinturel et al., 2017), but it is still
289 possible that these rates vary in particular regions of the brain and affect sleep. The role
290 of rRNA synthesis in *Drosophila* learning and memory has barely been explored, but our
291 work, together with that of Allen et al. (2018), indicates that the well-known requirement
292 for *de novo* protein synthesis during long-term memory consolidation (Jarome and
293 Helmstetter, 2014) includes increased synthesis of ribosomal RNA and protein.
294

295 **Novel neuronal role of Regnase-1 in sleep and memory**

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

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

312 Our study reveals that the expression of Regnase-1 changes after training, and
313 constitutive downregulation of Regnase-1 in ap α′/β′ neurons reduces sleep and causes
314 deficits in learning and memory consolidation. Whether acute downregulation of
315 Regnase-1 is necessary for sleep-dependent memory remains unclear, but it could play
316 a role by promoting the translation of specific transcripts. For instance, post-training
317 downregulation of Regnase-1 could release mRNAs that are usually targeted for decay
318 but are critical for memory consolidation. Constitutive loss of Regnase-1 impairs sleep-
319 independent memory and short-term memory, although the mechanisms are not known.
320 We note that ap α′/β′ neurons have a role in several aspects of memory consolidation as
321 well as short-term appetitive memory retrieval. Interestingly, constitutive knockdown of
322 Regnase-1 also reduces sleep developmentally and prevents sleep increase after
323 training in adult. While these could be independent effects, it is also possible that loss of
324 Regnase-1 affects the development of the relevant MB neurons, thereby impacting all
325 behaviors regulated by these neurons.

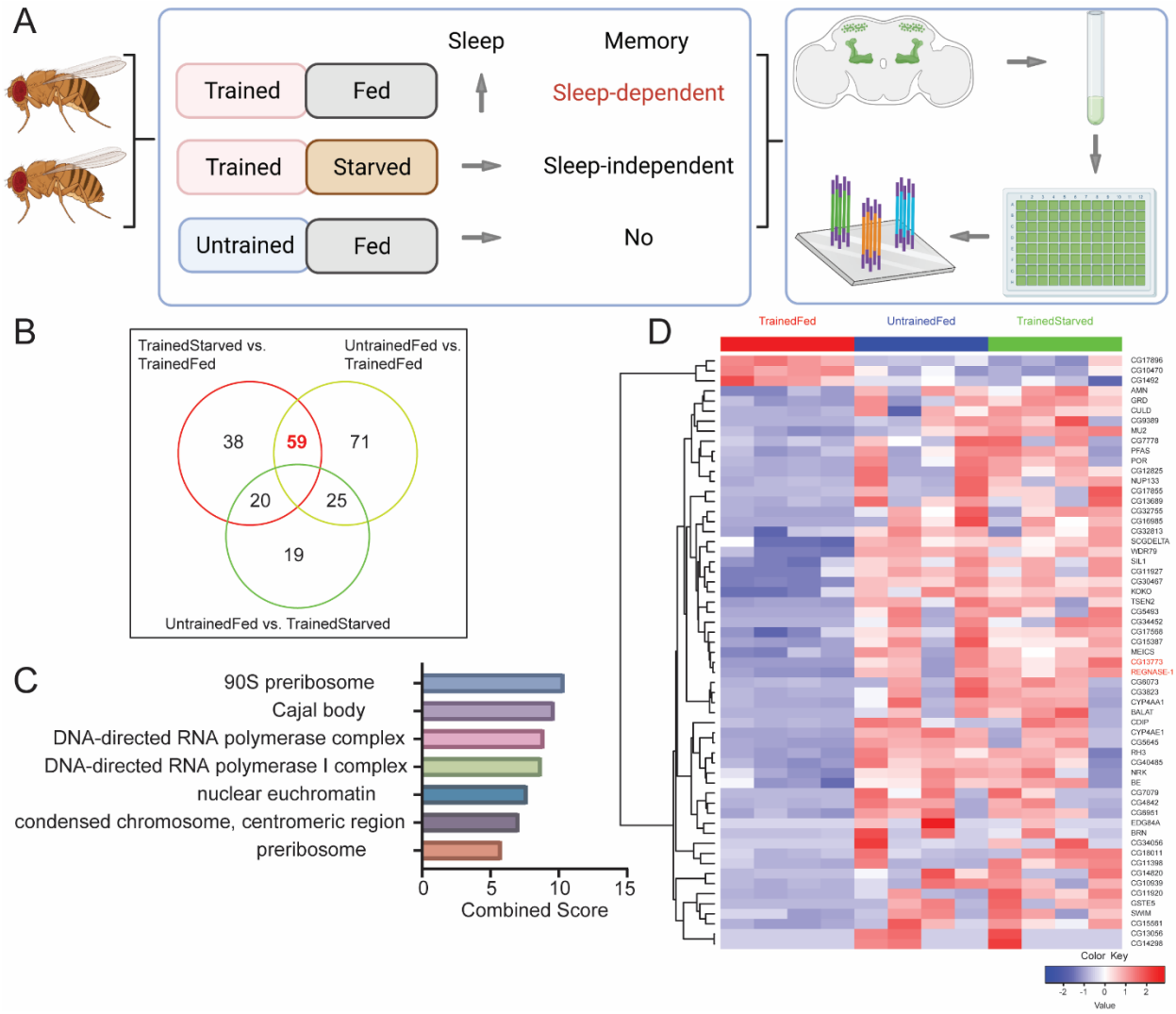
326 Our study of local molecular changes in ap α′/β′ neurons after training suggests
327 that ribosomal RNA transcription and mRNA translation might work in concert during the
328 consolidation of sleep-dependent memory. How sleep is involved in this boosted protein
329 synthesis process is unclear, but we suggest that RNA processing changes induce
330 sleep, which promotes translation necessary for consolidation of long-term memory.
331 However, these processes need to be teased apart experimentally. Overall, our findings

332 demonstrate a role of RNA processing in sleep and memory, providing a foundation for

333 future exploration of the mechanisms involved.

334

335 **Figure legends**



336

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

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

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

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

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

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

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

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

355

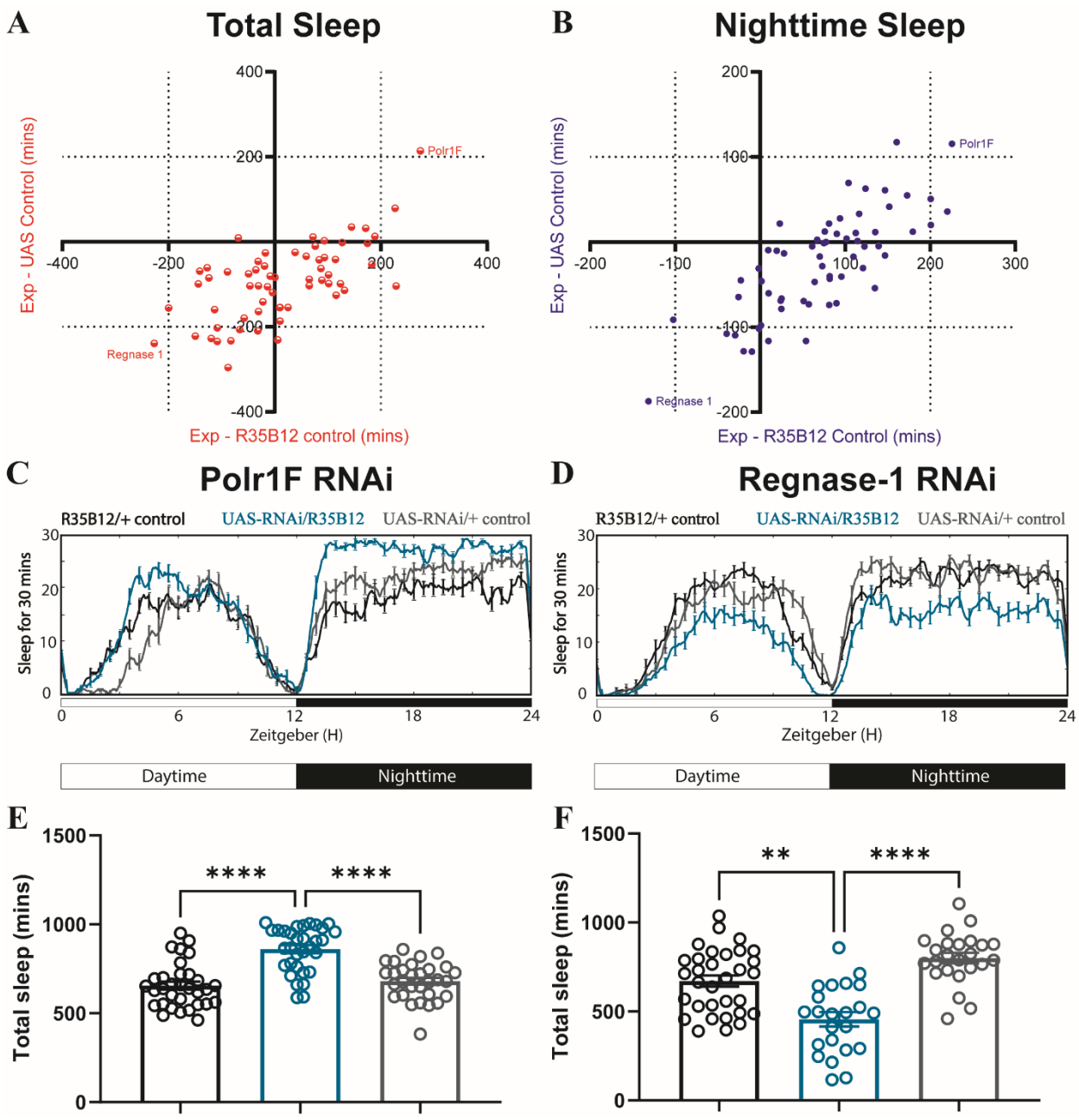


Figure 2. The sleep screen of differentially expressed genes identifies Polr1F and Regnase-1 as sleep-regulating genes. (A-B) Flies carrying the ap α′/β′ neuron driver R35B12-Gal4 were crossed with flies carrying UAS-RNAi constructs targeting DEGs identified from RNA-seq analysis. 5-7 days old female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

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

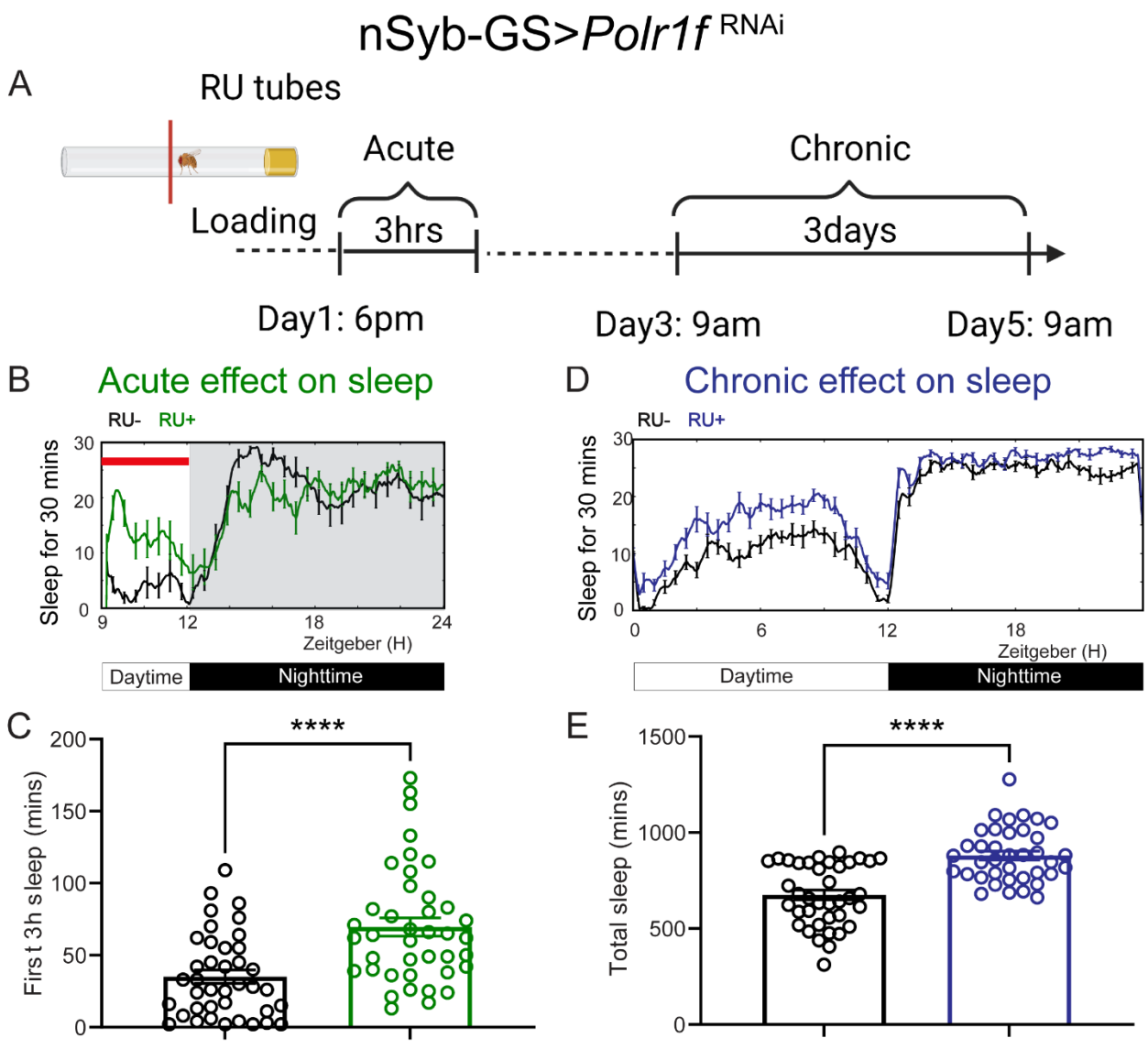
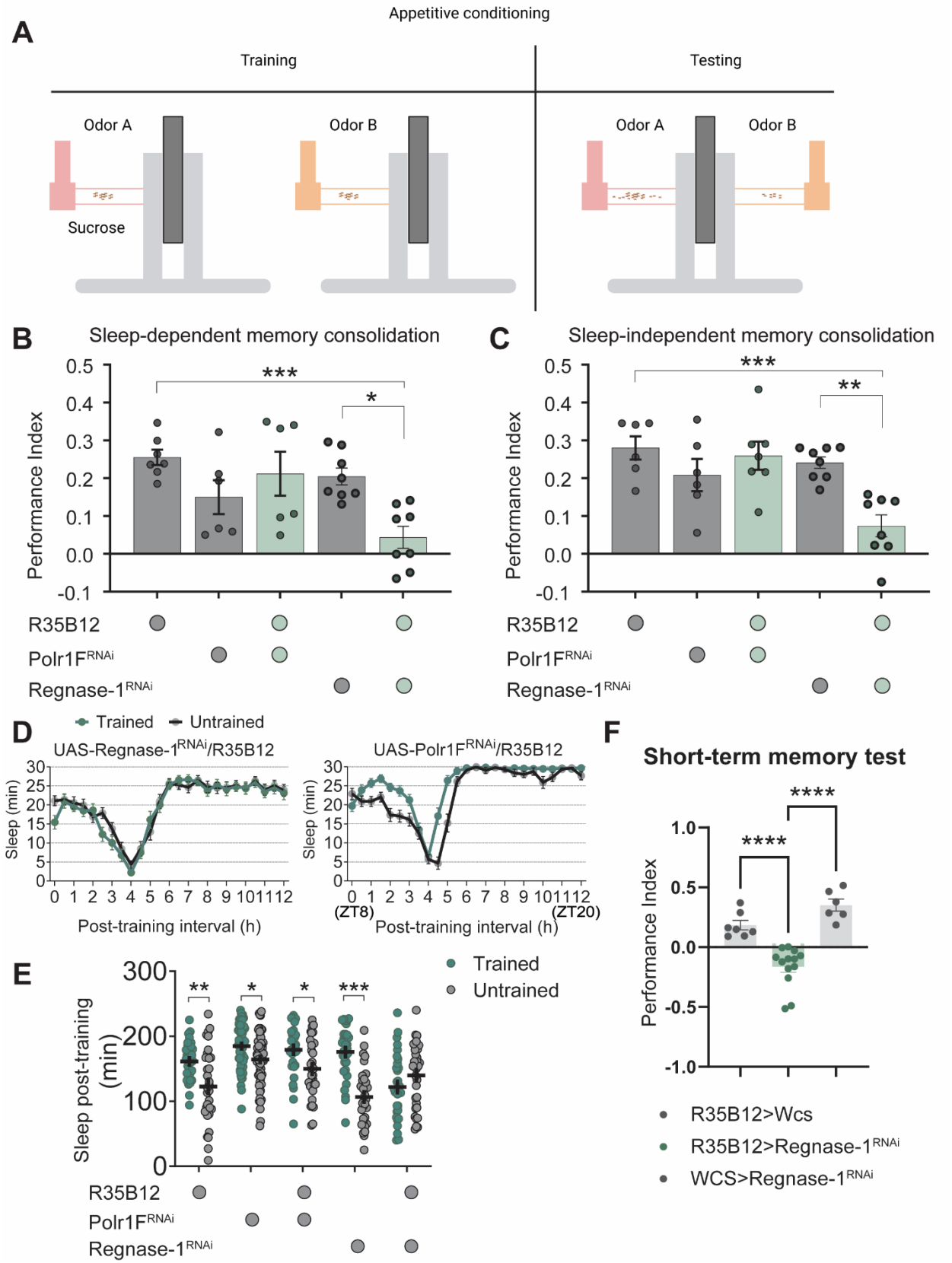


Figure 3. Acute and chronic effects of pan-neuronal knockdown of Polr1F on sleep in adult flies. (A) Schematic representation of transient and chronic sleep measurements in *nSyb-GS>polr1f*^{RNAi} flies. (B) Representative sleep traces and transient activity plot of flies expressing Polr1F RNAi under 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

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


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

389

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

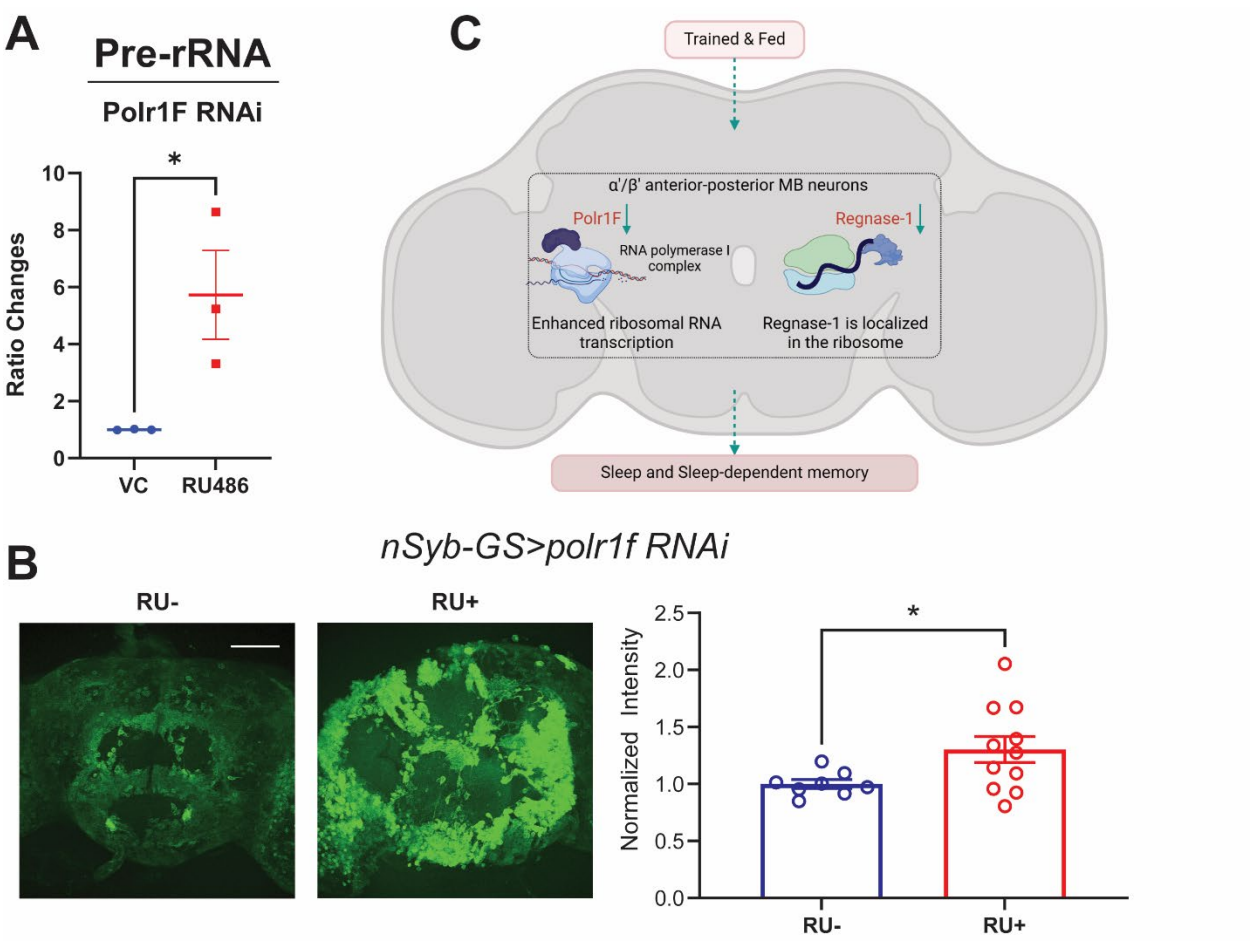


Figure 5. Knockdown of Polr1F results in high translation. (A) The *nSyb-GS>polr1f RNAi* flies exhibit a significant increase in pre-rRNA levels. (B) The ex-vivo puromycin immunostaining assay was used to measure translation in dissected whole brains. The results show that knockdown of Polr1F using the 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 major transcriptome features of ap α′/β′ neurons under trained and fed conditions, revealed in this manuscript. Two RNA processing genes Polr1F and

416 Regnase-1 are prominently downregulated during memory consolidation in trained and
417 fed flies, respectively. Polr1F is involved in regulating ribosomal RNA synthesis, and its
418 decrease in levels in trained and fed flies promotes sleep and translation. In contrast,
419 Regnase-1 is localized in the ribosome and involved in mRNA decay, and its
420 downregulation causes deficits in sleep and memory.

421

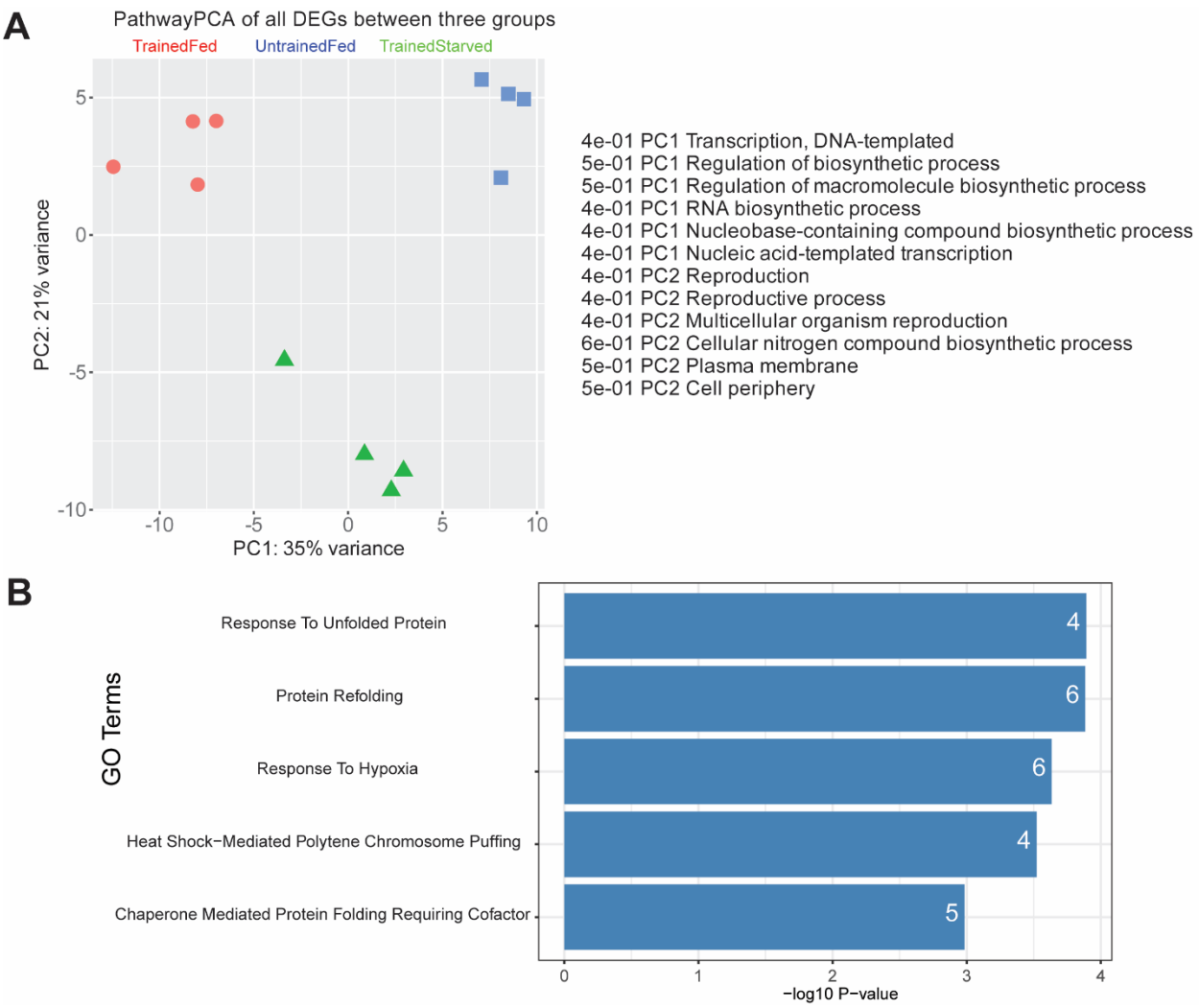
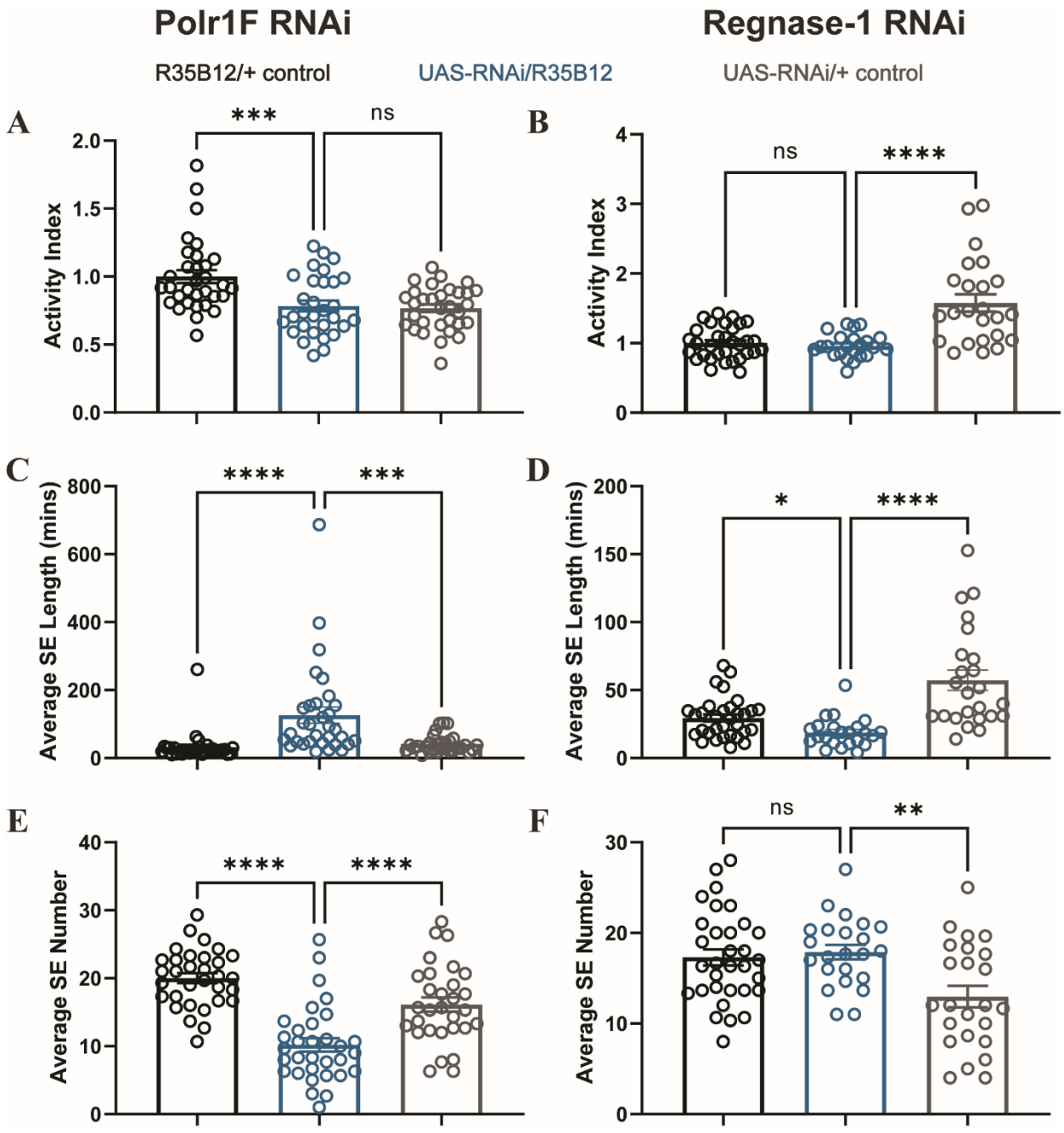


Figure 1 – supplemental figure 1. (A) PathwayPCA plot of all the differentially expressed genes (DEGs) across the three different conditions. The plot shows that genes responsible for PC1, which accounted for 35% of variance, largely encode proteins involved in transcription and biosynthesis, including 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.



431

432 **Figure 2 – supplemental figure 1. Effect of Polr1F and Regnase-1 knockdown on**

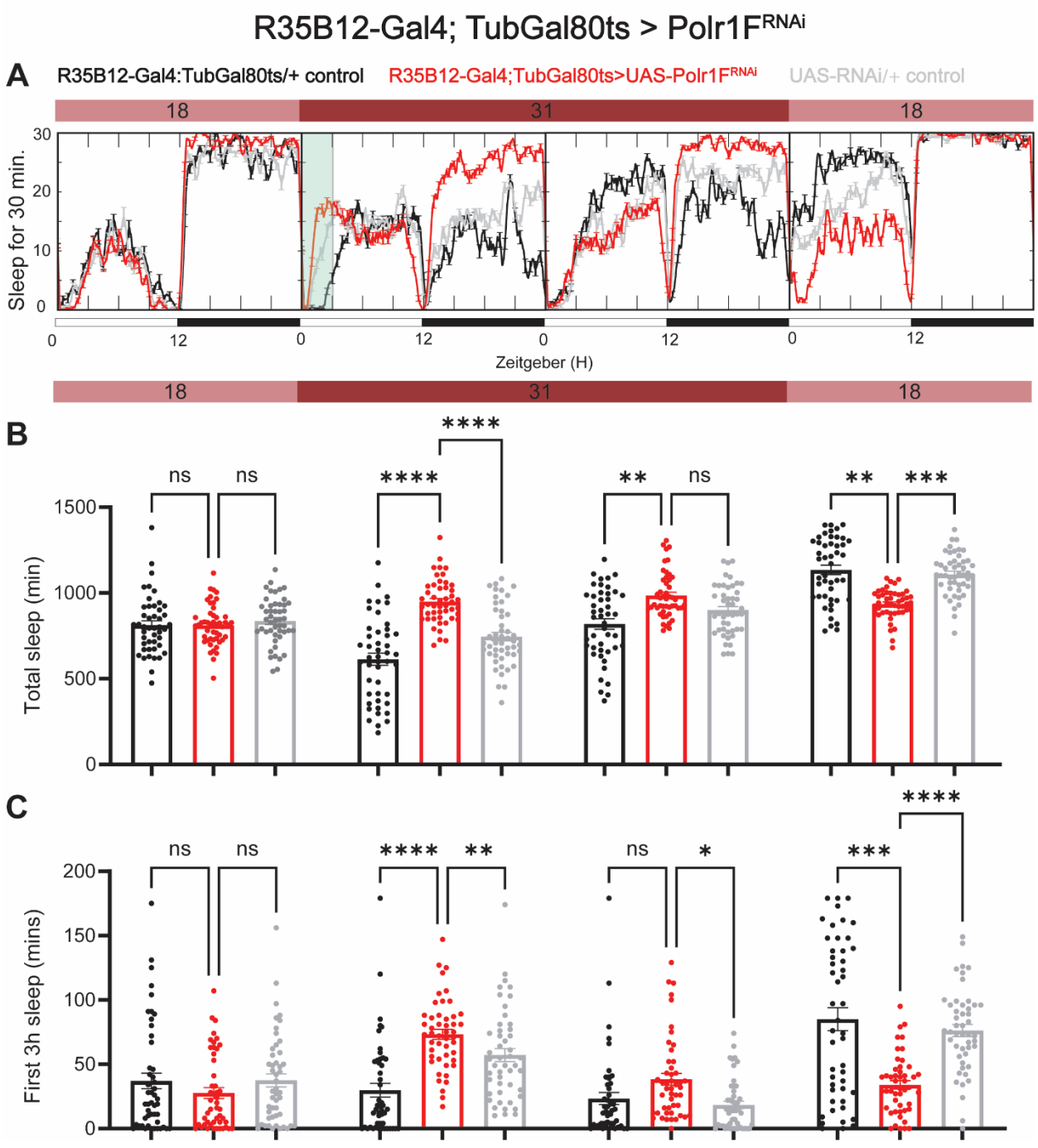
433 **activity and sleep architecture.** (A-B) Total activity of flies is not altered by knockdown

434 of Polr1F and Regnase-1. One-way ANOVA was used to calculate the p-values for each

435 comparison. (C-D) Knockdown of Polr1F significantly increases the nighttime average

436 sleep episode (SE) length in R35B12-Gal4>polr1F RNAi flies, while knockdown of

437 Regnase-1 reduces it in R35B12-Gal4>regnase1 RNAi flies. The p-values for each
438 comparison were calculated using the Kruskal–Wallis test with Dunn′s multiple
439 comparisons test. (E-F) Knockdown of Polr1F significantly reduces the nighttime
440 average sleep episode (SE) number in R35B12-Gal4>polr1FRNAi flies, but it is not
441 significant compared to control groups in R35B12-Gal4>regnase1RNAi flies. The p-
442 values for each comparison were calculated using the Kruskal–Wallis test with Dunn′s
443 multiple comparisons test. ns = not significant, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p <$
444 0.001, ****$p < 0.0001$.



445

446 **Figure 3 – supplemental figure 1. Adult specific knockdown of Polr1F promotes**

447 **sleep.** (A) Representative sleep traces of adult flies expressing Polr1F RNAi under the

448 control of R35B12-Gal4/Tubulin-gal80ts. At permissive temperature, Gal80ts inhibits

449 Gal4 activation of UAS; however, upon switching to restrictive temperature, Gal80ts is

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

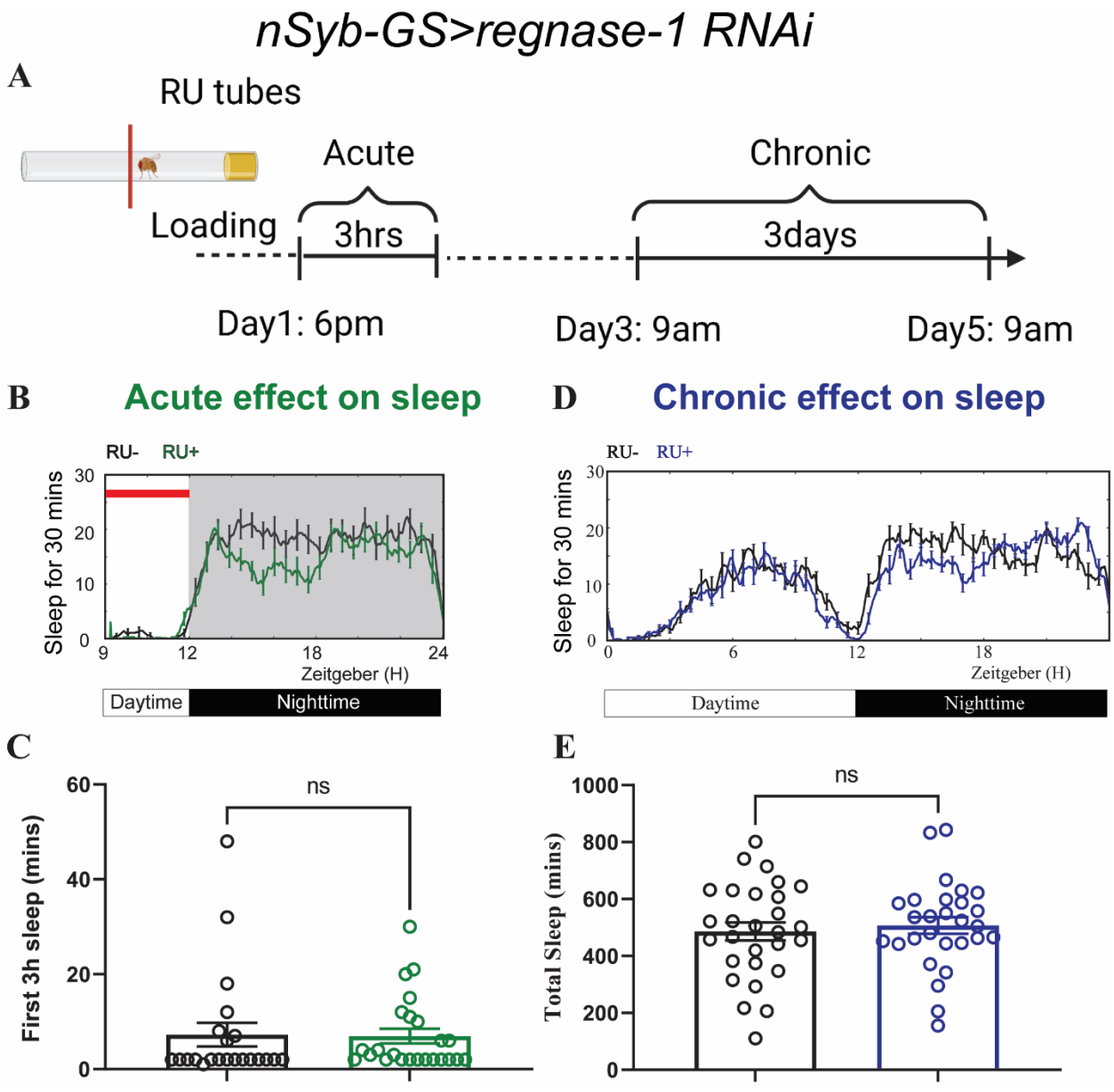


Figure 3 – supplemental figure 2. Acute and chronic effects of pan-neuronal knockdown of Regnase-1 on sleep in adult flies. (A) Schematic representation of the protocol for transient and chronic sleep measurements in *nSyb-GS>regnase-1 RNAi* flies. (B) Representative sleep traces plot of flies expressing 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

465 progeny flies were loaded into RU- or RU+ DAM tubes at ZT8-T9. Sleep was measured
466 starting at ZT9. N = 22-24 individual flies per replicate with data from two independent
467 replicates combined. The Mann-Whitney test was used to compare RU+ group and RU-
468 groups. (D) Representative average sleep traces of *nSyb-GS>regnase-1 RNAi* in the
469 RU- and RU+ DAM tubes for three consecutive days. Chronic sleep effects of
470 knockdown of Regnase-1 were measured based on sleep data from day 3 to day 5. (E)
471 Quantification of average total sleep of *nSyb-GS>regnase-1 RNAi* and controls in the
472 DAM tubes from (D). Unpaired t-test was used to compare between RU- and RU+
473 group. ns = not significant.
474

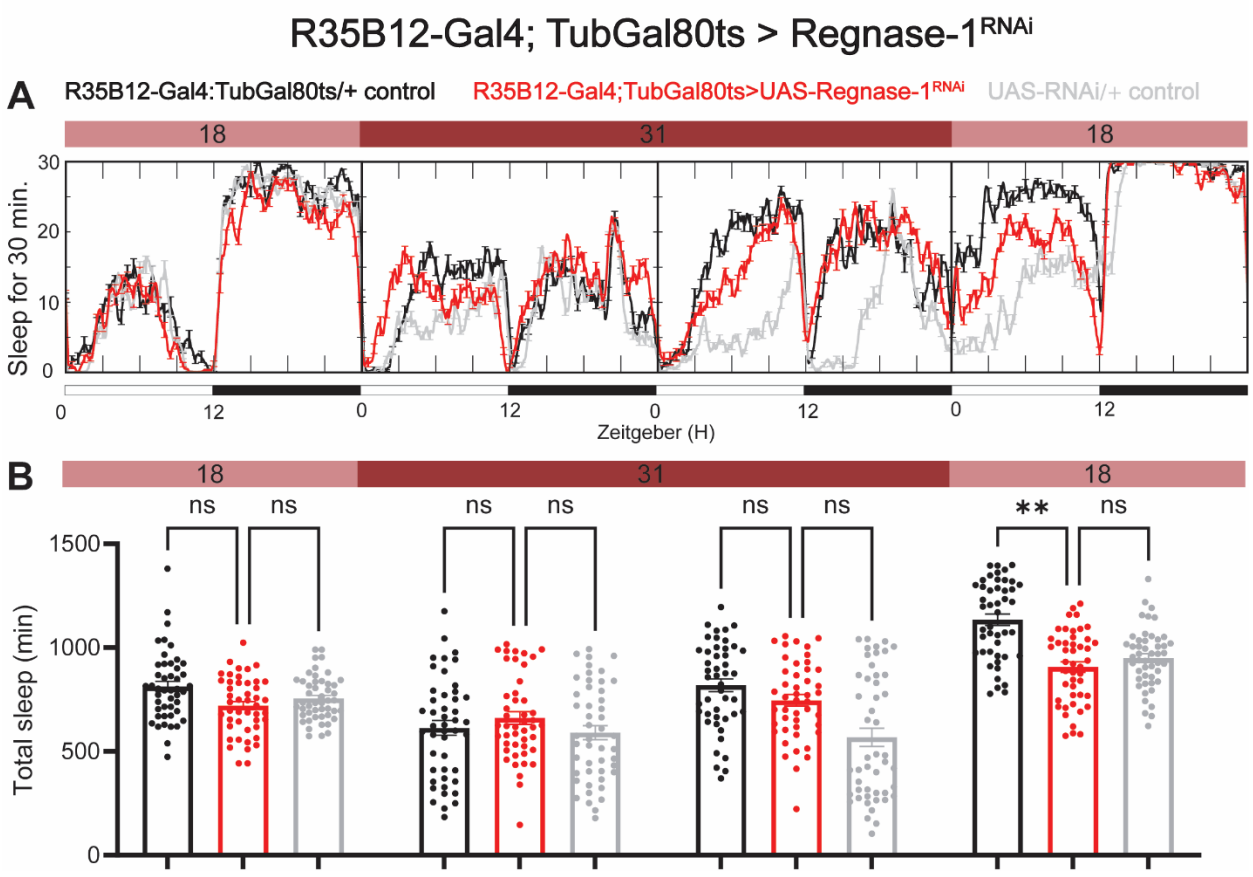


Figure 3 – supplemental figure 3. Adult specific knockdown of Regnase-1 has no effect on sleep. (A) Representative sleep traces of adult flies expressing Regnase-1 RNAi under the control of R35B12-Gal4/Tubulin-gal80ts. (B) Sleep was quantified over four consecutive days, with flies under permissive temperature on the first and last days and restrictive temperature on the second and third days. N = 46 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, $p > 0.05$, $*p < 0.05$, $**p < 0.01$.

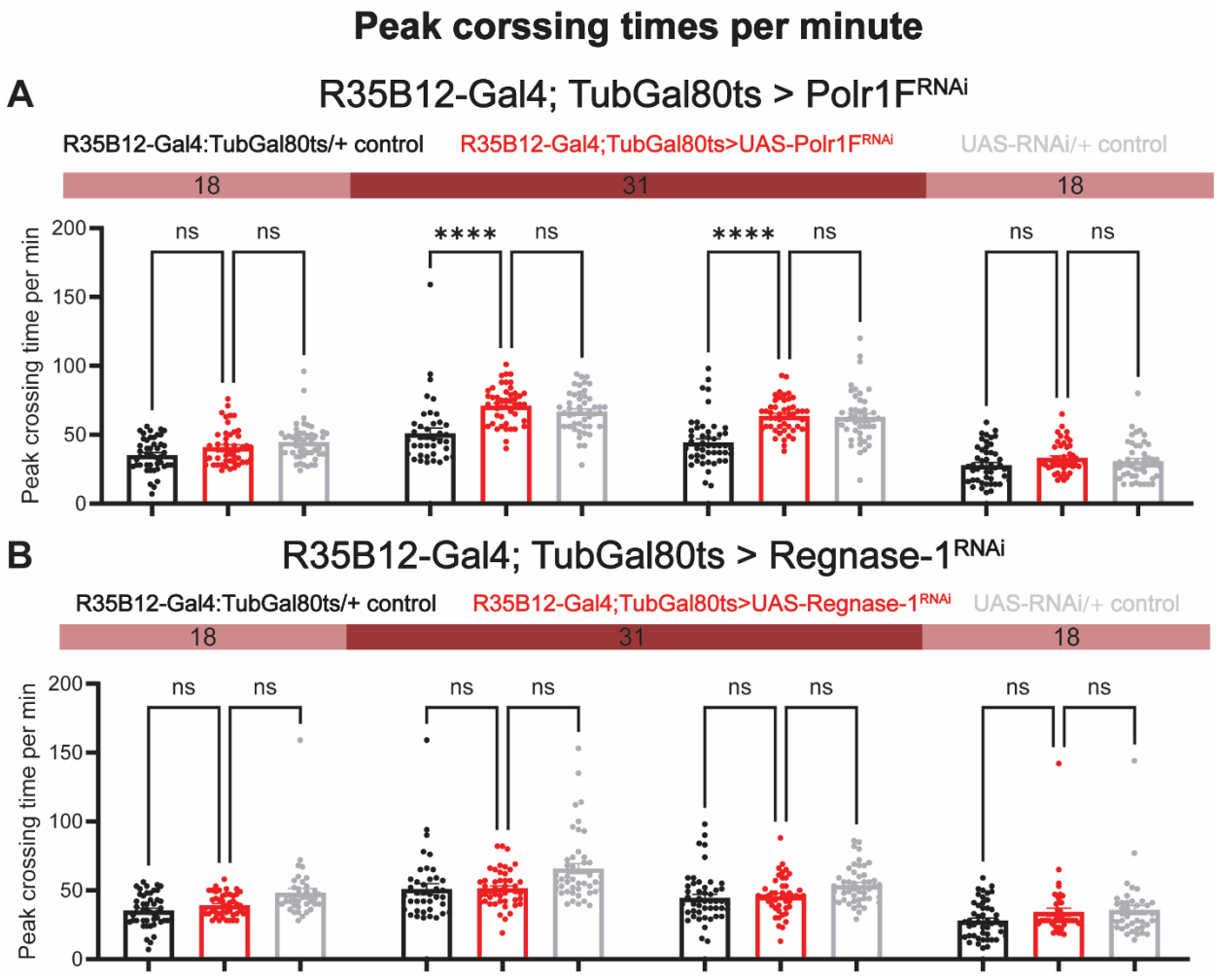
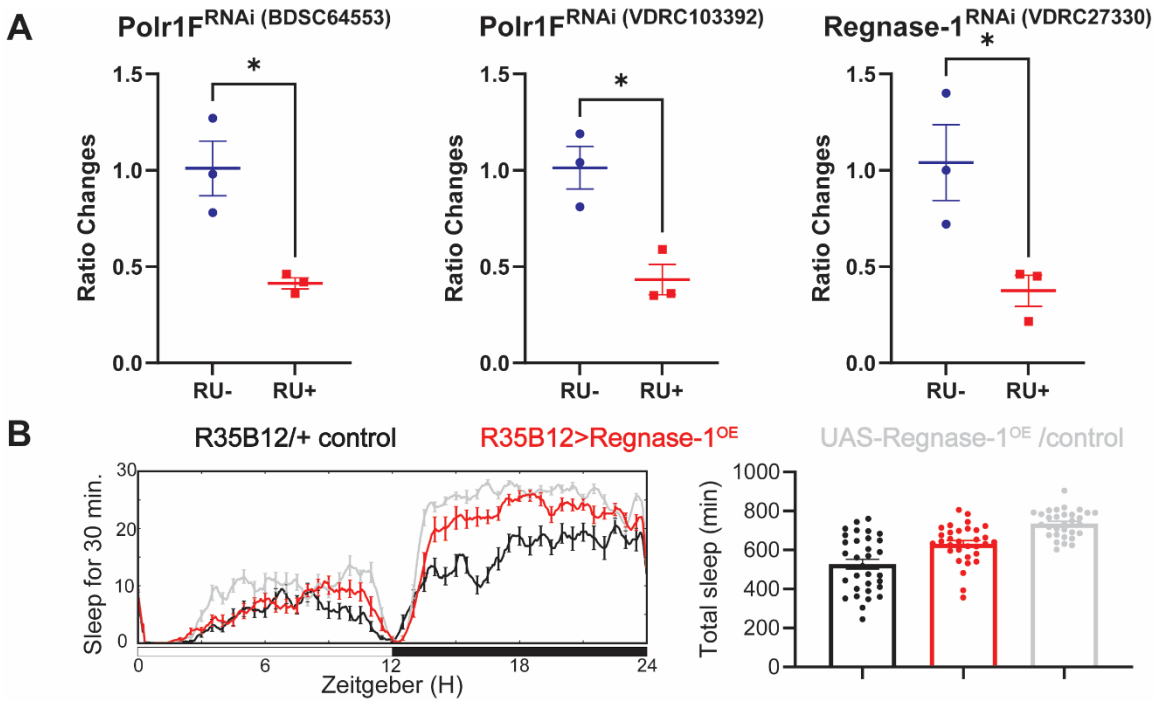


Figure 3 – supplemental figure 4. Adult specific knockdown of Polr1F or Regnase-1 has no effect on peak activity. (A) Maximal activity per minute for R35B12-Gal4; TubGal80ts>polr1F RNAi was quantified over four consecutive days, with flies under permissive temperature on the first and last days and restrictive 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, $p > 0.05$, *****$p < 0.0001$.



494

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

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

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

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

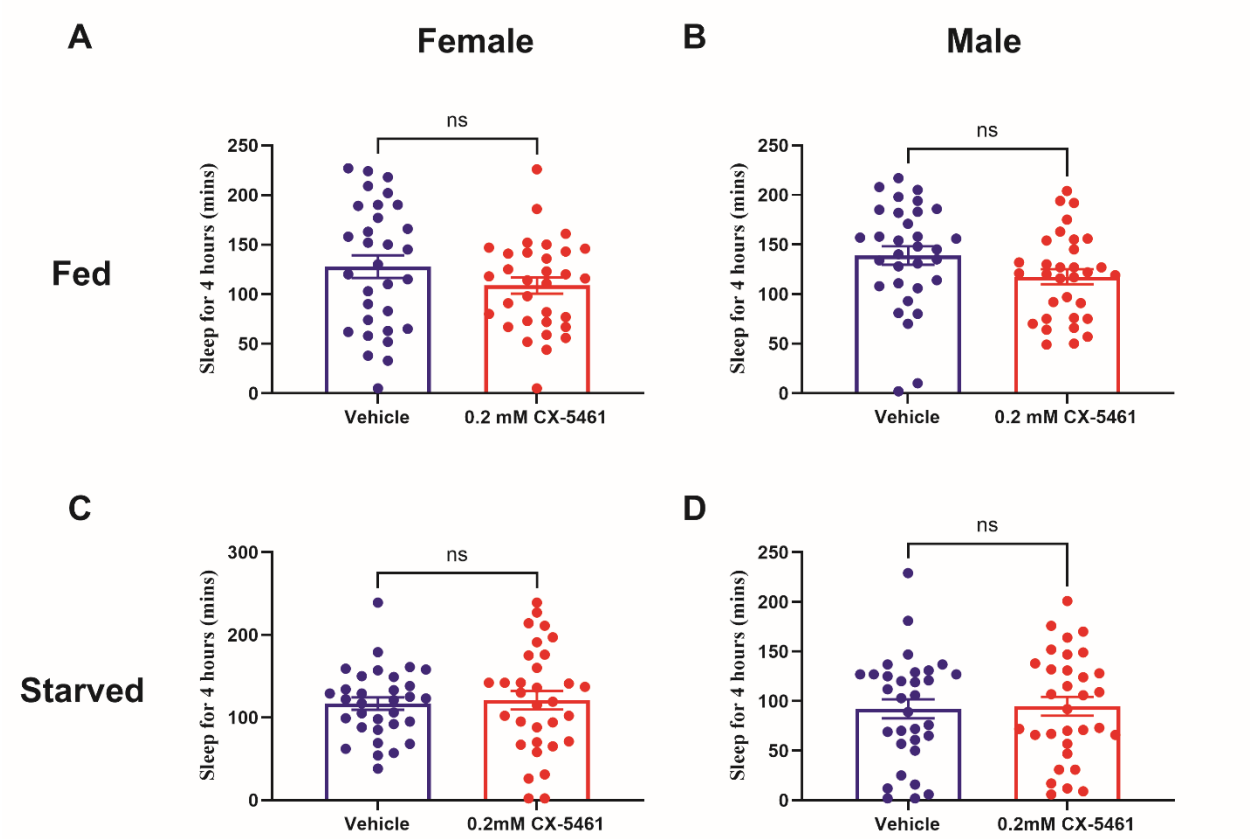
499 being dissected for qPCR. Welch's test was used for statistical analysis across all three

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

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

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

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



504

505 **Figure 5 – supplemental figure 1. rRNA inhibitor (CX-5461) feeding does not affect**

506 **sleep.** Feeding of the ribosome RNA inhibitor CX-5461 at a concentration of 0.2mM

507 does not affect sleep in fed or starved flies. Statistical analysis shows that there is no

508 significant difference between CX-5461-fed and control flies. The sample size is N=32

509 per group from two independent replicates. ns, not significant).

510 **Reference:**

511 Alberini CM, Kandel ER (2015) The Regulation of Transcription in Memory
512 Consolidation. Cold Spring Harbor Perspectives in Biology 7.

513 Allen KD, Regier MJ, Hsieh C, Tsokas P, Barnard M, Phatarpekar S, Wolk J, Sacktor
514 TC, Fenton AA, Hernández AI (2018) Learning-induced ribosomal RNA is
515 required for memory consolidation in mice—Evidence of differentially expressed
516 rRNA variants in learning and memory. PLOS ONE 13:e0203374.

517 Aso Y et al. (2014) Mushroom body output neurons encode valence and guide memory-
518 based action selection in Drosophila Luo L, ed. eLife 3:e04580.

519 Aviner R (2020) The science of puromycin: From studies of ribosome function to
520 applications in biotechnology. Comput Struct Biotechnol J 18:1074–1083.

521 Beckouët F, Mariotte-Labarre S, Peyroche G, Nogi Y, Thuriaux P (2011) Rpa43 and its
522 partners in the yeast RNA polymerase I transcription complex. FEBS Letters
523 585:3355–3359.

524 Cavallaro S, D'Agata V, Manickam P, Dufour F, Alkon DL (2002) Memory-specific
525 temporal profiles of gene expression in the hippocampus. Proceedings of the
526 National Academy of Sciences 99:16279–16284.

527 Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A (2013)
528 Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool.
529 BMC Bioinformatics 14:128.

530 Chouhan NS, Griffith LC, Haynes P, Sehgal A (2021) Availability of food determines the
531 need for sleep in memory consolidation. Nature 589:582–585.

532 Dag U, Lei Z, Le JQ, Wong A, Bushey D, Keleman K (2019) Neuronal reactivation
533 during post- learning sleep consolidates long-term memory in drosophila. eLife 8.

534 Deliu LP, Ghosh A, Grewal SS (2017) Investigation of protein synthesis in Drosophila
535 larvae using puromycin labelling. Biol Open 6:1229–1234.

536 Di Giorgio ML, Esposito A, Maccallini P, Micheli E, Bavasso F, Gallotta I, Vernì F,
537 Feiguin F, Cacchione S, McCabe BD, Di Schiavi E, Raffa GD (2017)
538 WDR79/TCAB1 plays a conserved role in the control of locomotion and
539 ameliorates phenotypic defects in SMA models. Neurobiol Dis 105:42–50.

540 Diekelmann S, Born J (2010) The memory function of sleep. Nat Rev Neurosci 11:114–
541 126.

542 Ge SX, Son EW, Yao R (2018) iDEP: an integrated web application for differential
543 expression and pathway analysis of RNA-Seq data. BMC Bioinformatics 19:534.

544 Herold N, Will CL, Wolf E, Kastner B, Urlaub H, Lührmann R (2009) Conservation of the
545 Protein Composition and Electron Microscopy Structure of Drosophila
546 melanogaster and Human Spliceosomal Complexes. Mol Cell Biol 29:281–301.

547 Hoedjes KM, Smid HM, Schijlen EG, Vet LE, van Vugt JJ (2015) Learning-induced gene
548 expression in the heads of two Nasonia species that differ in long-term memory
549 formation. BMC Genomics 16:162.

550 Jarome TJ, Helmstetter FJ (2014) Protein degradation and protein synthesis in long-
551 term memory formation. Frontiers in Molecular Neuroscience 7 Available at:
552 https://www.frontiersin.org/articles/10.3389/fnmol.2014.00061 [Accessed
553 September 28, 2022].

554 Krashes MJ, Keene AC, Leung B, Armstrong JD, Waddell S (2007) Sequential use of
555 mushroom body neuron subsets during drosophila odor memory processing.
556 Neuron 53:103–115.

557 Krashes MJ, Waddell S (2008) Rapid Consolidation to a radish and Protein Synthesis-
558 Dependent Long-Term Memory after Single-Session Appetitive Olfactory
559 Conditioning in Drosophila. J Neurosci 28:3103–3113.

560 Li H, Horns F, Wu B, Xie Q, Li J, Li T, Luginbuhl DJ, Quake SR, Luo L (2017)
561 Classifying Drosophila Olfactory Projection Neuron Subtypes by Single-Cell RNA
562 Sequencing. Cell 171:1206-1220.e22.

563 Liu X-X, Wang C, Huang S-F, Chen Q, Hu Y-F, Zhou L, Gu Y (2016) Regnase-1 in
564 microglia negatively regulates high mobility group box 1-mediated inflammation
565 and neuronal injury. Sci Rep 6:24073.

566 Mao R, Yang R, Chen X, Harhaj EW, Wang X, Fan Y (2017) Regnase-1, a rapid
567 response ribonuclease regulating inflammation and stress responses. Cell Mol
568 Immunol 14:412–422.

569 Marygold SJ, Alic N, Gilmour DS, Grewal SS (2020) In silico identification of Drosophila
570 melanogaster genes encoding RNA polymerase subunits. MicroPubl Biol
571 2020:10.17912/micropub.biology.000320.

572 Mino T, Murakawa Y, Fukao A, Vandenbon A, Wessels H-H, Ori D, Uehata T, Tartey S,
573 Akira S, Suzuki Y, Vinuesa CG, Ohler U, Standley DM, Landthaler M, Fujiwara T,
574 Takeuchi O (2015) Regnase-1 and Roquin Regulate a Common Element in
575 Inflammatory mRNAs by Spatiotemporally Distinct Mechanisms. Cell 161:1058–
576 1073.

577 Odom GJ, Ban Y, Colaprico A, Liu L, Silva TC, Sun X, Pico AR, Zhang B, Wang L,
578 Chen X (2020) PathwayPCA: an R/Bioconductor Package for Pathway Based
579 Integrative Analysis of Multi-Omics Data. Proteomics 20:e1900409.

580 Rasch B, Born J (2013) About Sleep's Role in Memory. Physiological Reviews 93:681.

581 Robles-Murguia M, Hunt LC, Finkelstein D, Fan Y, Demontis F (2019) Tissue-specific
582 alteration of gene expression and function by RU486 and the GeneSwitch
583 system. npj Aging Mech Dis 5:1–5.

584 Rogg M, Maier JI, Ehle M, Sammarco A, Schilling O, Werner M, Schell C (2022)
585 NUP133 Controls Nuclear Pore Assembly, Transcriptome Composition, and
586 Cytoskeleton Regulation in Podocytes. Cells 11:1259.

587 Roselli C, Ramaswami M, Boto T, Cervantes-Sandoval I (2021) The Making of Long-
588 Lasting Memories: A Fruit Fly Perspective. Frontiers in Behavioral Neuroscience
589 15.

590 Rothblum K, Hu Q, Penrod Y, Rothblum LI (2014) Selective Inhibition of rDNA
591 Transcription by a Small-Molecule Peptide That Targets the Interface between
592 RNA Polymerase I and Rrn3. Molecular Cancer Research 12:1586–1596.

593 Rutherford S, Barkema P, Tso IF, Sripada C, Beckmann CF, Ruhe HG, Marquand AF
594 (2023) Evidence for embracing normative modeling Baker CI, Constable T,
595 Esteban O, eds. eLife 12:e85082.

596 Seibt J, Frank MG (2012) Translation regulation in sleep. Commun Integr Biol 5:491–
597 495.

598 Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC, Imamichi T, Chang W
599 (2022) DAVID: a web server for functional enrichment analysis and functional
600 annotation of gene lists (2021 update). Nucleic Acids Res 50:W216–W221.

601 Shyu W-H, Lee W-P, Chiang M-H, Chang C-C, Fu T-F, Chiang H-C, Wu T, Wu C-L
602 (2019) Electrical synapses between mushroom body neurons are critical for
603 consolidated memory retrieval in Drosophila. PLoS Genet 15:e1008153.

604 Sinturel F, Gerber A, Mauvoisin D, Wang J, Gatfield D, Stubblefield JJ, Green CB,
605 Gachon F, Schibler U (2017) Diurnal Oscillations in Liver Mass and Cell Size
606 Accompany Ribosome Assembly Cycles. Cell 169:651-663.e14.

607 Tadi M, Allaman I, Lengacher S, Grenningloh G, Magistretti PJ (2015) Learning-Induced
608 Gene Expression in the Hippocampus Reveals a Role of Neuron -Astrocyte
609 Metabolic Coupling in Long Term Memory. PLoS One 10:e0141568.

610 Tanaka H, Arima Y, Kamimura D, Tanaka Y, Takahashi N, Uehata T, Maeda K, Satoh
611 T, Murakami M, Akira S (2019) Phosphorylation-dependent Regnase-1 release
612 from endoplasmic reticulum is critical in IL-17 response. Journal of Experimental
613 Medicine 216:1431–1449.

614 Thuriaux P, Mariotte S, Buhler J-M, Sentenac A, Vu L, Lee B-S, Nomura M (1995) Gene
615 RPA43 in Saccharomyces cerevisiae Encodes an Essential Subunit of RNA
616 Polymerase I (∗). Journal of Biological Chemistry 270:24252–24257.

617 Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K,
618 Satoh T, Mino T, Suzuki Y, Standley DM, Tsujimura T, Rakugi H, Isaka Y,
619 Takeuchi O, Akira S (2013) Malt1-Induced Cleavage of Regnase-1 in CD4+
620 Helper T Cells Regulates Immune Activation. Cell 153:1036–1049.

621 Wagner U, Kashyap N, Diekelmann S, Born J (2007) The impact of post-learning sleep
622 vs. wakefulness on recognition memory for faces with different facial
623 expressions. Neurobiology of learning and memory 87:679–687.

624 Wei J, Long L, Zheng W, Dhungana Y, Lim SA, Guy C, Wang Y, Wang Y-D, Qian C, Xu
625 B, Kc A, Saravia J, Huang H, Yu J, Doench JG, Geiger TL, Chi H (2019)
626 Targeting REGNASE-1 programs long-lived effector T cells for cancer therapy.
627 Nature 576:471–476.

628 Xu K, Zheng X, Sehgal A (2008) Regulation of Feeding and Metabolism by Neuronal
629 and Peripheral Clocks in Drosophila. Cell Metabolism 8:289–300.

630 Zhu L, Liao SE, Fukunaga R (2019) Drosophila Regnase-1 RNase is required for mRNA
631 and miRNA profile remodelling during larva-to-adult metamorphosis. RNA Biol
632 16:1386–1400.

633 Zimmerman JE, Rizzo W, Shockley KR, Raizen DM, Naidoo N, Mackiewicz M, Churchill
634 GA, Pack AI (2006) Multiple mechanisms limit the duration of wakefulness in
635 Drosophila brain. Physiological Genomics 27:337–350.

636

637 **Conflict of interest statement**

638 The authors declare no competing interests.

639 **Acknowledgments**

640 This work was supported by Howard Hughes Medical Institute (HHMI). We thank all
641 members of the Sehgal lab for reagents, comments and support, especially rotation
642 undergraduate Arielle Ketchum for help with molecular cloning. We thank Hongjie Li of
643 Liqun Luo lab with technical assistance and protocol sharing from Stanford University.
644 We thank Ryuya Fukunaga lab for kindly sharing with us the Regnase-1 overexpression
645 line.

646 **Author contributions**

647 Conceptualization, Y.J.L., N.S.C. and A.S.; Methodology, Y.J.L., N.S.C., S.Z., S.B.N.
648 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
649 Z.F.Y.; Formal analysis, Y.J.L., N.S.C. and R.M.; Investigation, Y.J.L. and N.S.C.;
650 Resources, Y.J.L., N.S.C., S.Z., R.M., J.S., A.K. and Z.F.Y.; Data Curation, Y.J.L.,
651 N.S.C. and R.M.; Writing – Original Draft, Y.J.L. and N.S.C. Writing – Review & Editing,
652 Y.J.L., N.S.C. and A.S. Visualization, Y.J.L., N.S.C. and R.M.; Supervision, Y.J.L.,
653 N.S.C. and A.S.; Project administration, A.S.; Funding acquisition, A.S.

654

655

656 **Materials and Methods**

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

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

659

660 **Key resource table**

661 See Appendix 1.

662

663 **Fly stock and maintenance**

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

668

669 **Behavior measurement in *Drosophila***

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

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

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

724 (https://warwick.ac.uk/fac/sci/lifesci/research/libinorm/) using the GENCODE reference
725 genome. Raw count and TPM were used separately in further analysis. Raw count data
726 were analyzed by IDEP v0.95 to identify genes expressed differentially between three
727 conditions (Ge et al., 2018). We filtered out low-expressed genes using a cutoff of
728 CPM>0.5, at least detected in 3 independent samples, and treated missing values as
729 gene median. Regularized log transformation was used to transform raw count data for
730 clustering and PCA. Differentially expressed genes were identified using DESeq2 with
731 an FDR cutoff of 0.1 and minimum fold change of 2.
732

733 **Puromycin assay and imaging**

734 We developed a puromycin assay to measure the rate and localization of nascent
735 peptide synthesis in the fly brain, and similar method has been described in the fly
736 larvae (Deliu et al., 2017). Fly brains were dissected in Schneiders′ medium and
737 incubated with puromycin for 40 min *in vitro* to allow puromycin to incorporate into the
738 newly synthesized peptide. Subsequently, using an anti-puromycin antibody, standard
739 immunostaining protocols were applied to detect the number and position of newly
740 synthesized puromycin-tagged peptides (Aviner, 2020), which provided an estimate of
741 translation rate. The brains were then imaged with a 20X oil immersion confocal
742 microscope with a resolution of 1024*1024. The GFP intensity of the images was then
743 measured by Fiji software (ImageJ) and the average intensity of the samples was
744 analyzed for comparison.
745

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

747 CX-5461 was mixed with 2% agar and 5% sucrose to make fly motor tubes at a final
748 concentration of 0.2 mM. Flies are loaded into these CX-5461 tubes or vehicle control
749 tubes 4 hours before light turns off and transient sleep changes are measured.
750

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

752 10 flies′ brains from each group were dissected for RNA extraction using Qiagen's
753 RNeasy Plus Mini Kit. Total RNA was then reverse transcribed to cDNA by using
754 random hexamers and Superscript II from Invitrogen. qPCR was performed using
755 SYBR-green master mix and oligonucleotide information is provided in the Key
756 Resources table. Relative gene expression was analyzed using the ΔΔCt method.
757

758 **Statistical Analysis**

759 Fly sleep behavioral data extracted from Pysolo was analyzed by GraphPad Prism
760 (https://www.graphpad.com/). Data from different replicates were pooled directly and
761 first tested for normality using D'Agostino-Pearson and Shapiro-Wilk tests. For normally
762 distributed data, unpaired parametric Student's t-test is used for two-sample
763 experiments and one-way ANOVA with Turkey post hoc test for three-sample or more
764 experiments. Non-normally distributed data were analyzed using nonparametric tests,
765 the Mann-Whitney test for two-sample experiments and the Kruskal-Wallis test with
766 Dunn's multiple comparisons test for three or more samples experiments. For all
767 graphs, unless otherwise stated, data are presented as mean and standard error of the
768 mean (SEM) and statistical significance was accepted for p value < 0.05.
769

770 **Data availability**

771 Sequencing data is available at NCBI BioProject with accession number

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

773 supporting files.

774

775 **Supplemental information**

776 Supplemental information includes 8 figures and 1 table.