

A feedback loop between heterochromatin and the nucleopore complex controls germ-cell to oocyte transition during *Drosophila* oogenesis

Kahini Sarkar¹, Noor M Kotb^{1,2}, Alex Lemus¹, Elliot T Martin¹, Alicia McCarthy^{1,3}, Justin Camacho¹, Ayman Iqbal¹, Alex M. Valm¹, Morgan A Sammons¹, Prashanth Rangan^{1*}

¹Department of Biological Sciences/RNA Institute, University at Albany SUNY, Albany, NY 12222

²Department of Biomedical Sciences, The School of Public Health, University at Albany SUNY, Albany, NY 12222

³Current address: 10x Genomics Headquarters, 6230 Stoneridge Mall Rd, Pleasanton, CA 94588

*Corresponding Author and Lead Contact: prangan@albany.edu

Summary

21 Germ cells differentiate into oocytes that become totipotent upon fertilization. How the highly
22 specialized oocyte acquires this distinct cell fate is poorly understood. During *Drosophila*
23 oogenesis, H3K9me3 histone methyltransferase SETDB1 translocates from the cytoplasm to the
24 nucleus of germ cells concurrent with oocyte specification. Here, we discovered that nuclear
25 SETDB1 is required to silence a cohort of differentiation-promoting genes by mediating their
26 heterochromatinization. Intriguingly, SETDB1 is also required for the upregulation of 18 of the ~30
27 nucleoporins (Nups) that comprise the nucleopore complex (NPC). NPCs in turn anchor SETDB1-
28 dependent heterochromatin at the nuclear periphery to maintain H3K9me3 and gene silencing in
29 the egg chambers. Aberrant gene expression due to loss of SETDB1 or Nups results in loss of
30 oocyte identity, cell death and sterility. Thus, a feedback loop between heterochromatin and NPCs
31 promotes transcriptional reprogramming at the onset of oocyte specification that is critical to
32 establish oocyte identity.

Introduction

36 Germ cells give rise to gametes that upon fertilization launch the next generation (Cinalli et al.,
37 2008; Seydoux and Braun, 2006; Spradling et al., 2011). In the gonad, germ cells become
38 germline stem cells (GSCs) that self-renew and differentiate to give rise to sperm or an oocyte
39 (Gilboa and Lehmann, 2004; Kershner et al., 2013; Ko et al., 2010; Lesch and Page, 2012; Reik
40 and Surani, 2015; Seydoux and Braun, 2006). The oocyte, upon fertilization or by
41 parthenogenesis, can differentiate into every cell lineage in the adult organism and thus has a
42 capacity to be totipotent (Ben-Ami and Heller, 2005; Lehmann, 2012; Riparbelli et al., 2017; Yuan
43 and Yamashita, 2010). The gene regulatory mechanisms that enable the transition from germ
44 cells to oocytes are not fully understood.

45

46 *Drosophila* has a well-characterized transition from germline stem cell (GSC) to an oocyte
47 (Dansereau and Lasko, 2008; Gilboa and Lehmann, 2004; Spradling et al., 2011; Allan C
48 Spradling, 1993). *Drosophila* ovaries comprise individual units called ovarioles that house the
49 GSCs in a structure called the germarium (**Figure 1A-A1**) (Lehmann, 2012; Xie and Spradling,
50 2000). GSC division results in a new GSC (self-renewal) and a cystoblast, which differentiates via
51 incomplete mitotic divisions, giving rise to 2-, 4-, 8- and 16-cell cysts (**Figure 1A1**) (Chen and
52 McKearin, 2003a, 2003b; Xie, 2013). One of these 16 cells is specified as the oocyte whereas
53 the other 15 cells become nurse cells (Huynh and St Johnston, 2004; Koch et al., 1967; Navarro
54 et al., 2004). Somatic cells envelop the nurse cells and the specified oocyte to form an egg
55 chamber (**Figure 1A1**) (Xie and Spradling, 2000). The nurse cells produce mRNAs, called
56 maternal mRNAs, that are deposited into the specified oocyte mediated by an RNA binding
57 protein, Egalitarian (Egl) (Blatt et al., 2020; Kugler and Lasko, 2009; Lilly and Spradling, 1996;
58 Mach and Lehmann, 1997; Navarro et al., 2004; A C Spradling, 1993). An inability to specify or
59 maintain the oocyte fate leads to death of the egg chamber mid-oogenesis and, in turn, sterility
60 (Blatt et al., 2021; Navarro et al., 2004).

61

62 The transition from GSC to an oocyte requires dynamic changes in gene expression that promote
63 progressive differentiation (Flora et al., 2017). Once a GSC gives rise to the cystoblast, it
64 expresses differentiation factor Bag of marbles (Bam), promoting its differentiation to an 8-cell
65 cyst (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). In the 8-cell cyst, the
66 expression of the RNA binding fox-1 homolog 1 (Rbfox1) is required to mediate transition into the
67 16-cell cyst stage, allowing for an oocyte to be specified (Carreira-Rosario et al., 2016).
68 Translation of Rbfox1 requires increased levels of ribosomal small subunit protein 19 (RpS19)
69 accomplished in part by expression of the germline specific paralog *RpS19b* in the
70 undifferentiated and early differentiating stages (McCarthy et al., 2019). During differentiation,
71 the germline also initiates meiotic recombination mediated by the synaptonemal complex
72 consisting of proteins such as Sisters Unbound (Sunn), Corona (Cona) and Orientation Disruptor
73 (Ord) (Ables, 2015; Cahoon and Hawley, 2016; Hughes et al., 2018; Orr-Weaver, 1995; Page and
74 Hawley, 2001). More than one cell in the cyst stage initiates recombination but as oocyte
75 differentiation proceeds, only the specified oocyte retains the synaptonemal complex (**Figure**
76 **1A1**) (Ables, 2015; Orr-Weaver, 1995; Page and Hawley, 2001). After oocyte-specification, the
77 levels of mRNAs encoding *RpS19b* and some synaptonemal complex proteins are diminished,
78 suggesting early oogenesis genes are no longer expressed (McCarthy et al., 2019). How the
79 expression of these early oogenesis genes is attenuated is not known.

80

81 In *Drosophila*, the SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) (also
82 called Eggless) is required for deposition of gene silencing Histone H3 Lysine 9 trimethylation
83 (H3K9me3) marks and heterochromatin formation (Clough et al., 2014, 2007; Rangan et al.,
84 2011; Yoon et al., 2008). SETDB1 is expressed throughout *Drosophila* oogenesis, but as the
85 oocyte is specified, it shifts from a cytoplasmic to predominantly nuclear localization (Clough et
86 al., 2007). A conserved cofactor called Windei (Wde) is required for either nuclear translocation,
87 nuclear stability, or targeting of SETDB1 to its target loci (Koch et al., 2009; Osumi et al., 2019).
88 Loss of *SETDB1* during germline development results in an accumulation of undifferentiated cells

89 (Rangan et al., 2011; Smolko et al., 2018). In addition, loss of *SETDB1* and *wde* also result in egg
90 chambers that do not grow in size and die mid-oogenesis (Clough et al., 2014; Koch et al., 2009).
91 *SETDB1* is known to be required for silencing transposons and male-specific transcripts in the
92 female germline (Czech et al., 2018; Rangan et al., 2011; Smolko et al., 2018). However, neither
93 the upregulation of transposons nor male-specific genes in female germline result in egg
94 chambers that do not grow in size (Malone et al., 2009; Shapiro-Kulnane et al., 2015; Smolko et
95 al., 2020). Together these data suggests that *SETDB1* silences a yet-unidentified group of genes
96 to promote oogenesis.

97
98 Here, we find that genes that are expressed in early stages of oogenesis, including genes that
99 promote oocyte differentiation and synaptonemal complex formation, are silenced upon oocyte
100 specification, via a feedback loop between *SETDB1*-mediated heterochromatin and the
101 nucleopore complex (NPC). Inability to silence these differentiation-promoting genes due to loss
102 of either *SETDB1* or members of the NPC results in loss of oocyte identity and death. Several
103 aspects of germ cell differentiation have been studied and have been implicated in loss of fertility
104 in sexually reproducing organisms. Our work indicates that a previously unappreciated broad
105 transcriptional reprogramming silences critical aspects of the germ cell differentiation program at
106 the onset of oocyte specification and is essential to promote oocyte identity.

107
108 **Results**
109

110 ***SETDB1* promotes silencing of *RpS19b* reporter at the onset of oocyte specification**

111 We hypothesized that the expression of early oogenesis mRNAs such as *RpS19b* is silenced
112 upon oocyte specification. To monitor *RpS19b* expression, we used a reporter that expresses an
113 *RpS19b*-GFP fusion from the endogenous *RpS19b* promoter. This *RpS19b*-GFP shows high
114 expression in the germarium and attenuated expression post-oocyte specification and in the
115 subsequent egg chambers, consistent with its endogenous *RpS19b* mRNA expression pattern
116 (**Figure 1B-C1, G**) (Jevitt et al., 2020; McCarthy et al., 2019).

117
118 Using a previously characterized hemagglutinin (HA) tagged endogenous *SETDB1*, we found that
119 a large fraction of *SETDB1* translocates from the cytoplasm to the nucleus concurrent with oocyte
120 specification (**Figure S1A-A3**) (Seum et al., 2007). To test if *SETDB1* is required for the silencing
121 of *RpS19b* (Clough et al., 2014, 2007), we performed germline knockdown (GKD) of *SETDB1*, in
122 the background of *RpS19b*-GFP reporter. We detected the germline, *RpS19b*-GFP, and
123 spectrosomes/fusomes/somatic cell membrane in ovaries by immunostaining for Vasa, GFP, and
124 1B1, respectively (Lasko and Ashburner, 1988; Zaccai and Lipshitz, 1996). We found that,
125 compared to the control, GKD of *SETDB1* resulted in ectopic *RpS19b*-GFP protein expression in
126 the differentiated egg chambers without affecting levels in the undifferentiated stages (**Figure 1C-**
127 **G; Figure S1B**). Thus, *SETDB1* is required for repression of *RpS19b*-GFP reporter in the
128 differentiated egg chambers.

129
130 To determine if nuclear *SETDB1* was required to repress *RpS19b*-GFP post-oocyte specification,
131 we depleted *wde* in the germline and independently assayed for *SETDB1* nuclear localization,
132 H3K9me3, and *RpS19b*-GFP (**Figure S1C**). GKD of *wde* resulted in loss of nuclear *SETDB1* in

133 the differentiated stages of oogenesis without affecting cytoplasmic levels in the undifferentiated
134 stages (**Figure S1D-F**). Whereas GKD of *SETDB1* reduced H3K9me3 throughout oogenesis,
135 GKD of *wde* reduced H3K9me3 only in the differentiated egg chambers but not in the
136 undifferentiated stages (**Figure S1G-J**). We found that GKD of *wde*, like GKD of *SETDB1*, results
137 in ectopic *RpS19b-GFP* protein expression in the egg chambers without affecting levels in the
138 undifferentiated stages (**Figure 1C-G**). In addition to upregulation of *RpS19b-GFP*, GKD of both
139 *SETDB1* and *wde* resulted in egg chambers that did not grow in size and died mid-oogenesis as
140 previously reported (**Figure S1K**) (Clough et al., 2014; Koch et al., 2009). Thus, repression of the
141 *RpS19b-GFP* reporter in the differentiated egg chambers requires nuclear *SETDB1*.
142

143 **SETDB1 and Wde repress genes that are primarily expressed prior to oocyte specification**
144 To determine if *SETDB1* and *Wde* repress other differentiation-promoting genes in addition to
145 *RpS19b*, we performed RNA Sequencing (RNA-seq). We compared ovaries from *SETDB1*- and
146 *wde*- GKD flies to ovaries from wild-type (WT) flies, including young virgin flies which lack late-
147 stage egg chambers. Principal component analysis of the RNA-seq data revealed that *SETDB1*
148 and *wde* ovary transcriptomes closely resembles young virgin WT rather than adult WT (**Figure**
149 **S2A**). Using a 1.5-fold cut off (Fold Change (FC) \geq 1.5) and False Discovery Rate (FDR) <0.05 ,
150 we found that compared to young virgin WT control, 2316 genes were upregulated and 1972 were
151 downregulated in *SETDB1* GKD ovaries, and 1075 genes were upregulated and 442 were
152 downregulated in *wde*-GKD ovaries (**Figure 2A-B**) (**Supplemental Table 1**). Moreover,
153 comparing *wde*- to *SETDB1*- GKD ovaries showed significant overlap of the upregulated (80%)
154 and downregulated (75%) transcripts, suggesting that *SETDB1* and *Wde* co-regulate a cohort of
155 genes during oogenesis (**Figure 2C**; **Figure S2B**).
156

157 *SETDB1* and *Wde* are known to repress gene expression, thus we first focused on mRNAs with
158 increased levels in the GKD ovaries (Clough et al., 2014; Osumi et al., 2019). Gene Ontology
159 (GO) analysis of the shared upregulated RNAs indicated that many were genes involved in
160 differentiation (**Figure 2D**). Among the upregulated RNAs was *RpS19b*, validating our initial
161 screen, as well as genes that promote synaptonemal complex formation such as *sunn*, *ord* and
162 *conA* (**Figure 2E**; **Figure S2C-E**). In addition, the *blanks* mRNA, which is highly expressed only
163 in GSCs, cystoblasts and early cysts of WT, was upregulated and ectopically expressed in the
164 egg chambers of *SETDB1*- and *wde*-GKD ovaries (**Figure 2F**; **Figure S2F-I**) (Blatt et al., 2021).
165 *Blanks* is a component of a nuclear siRNA pathway that has critical roles in the testis but does
166 not have any overt function during oogenesis (Gerbasi et al., 2011). Thus, *SETDB1* and *wde*
167 repress a cohort of RNAs that are either critical for transition from GSC to an oocyte or merely
168 expressed during early oogenesis.
169

170 To determine when during oogenesis *SETDB1* and *Wde* act to repress genes, we analyzed
171 available RNA-seq libraries that were enriched for GSCs, cystoblasts, and cysts, early egg
172 chambers and late-stage egg chambers (McCarthy et al., 2019). We found that *SETDB1/wde*-
173 regulated RNAs decreased after the cyst stages and their levels were attenuated in the later
174 stages of oogenesis compared to non-targets (**Figure 2G**, **Figure S2J-L**) (McCarthy et al., 2019).
175 This reduction did not happen in absence of *SETDB1* and *wde* (**Figure 2G**). RNA *in situ* analysis
176 of *blanks*, and *RpS19b* revealed that these mRNAs are present in the early stages of oogenesis

177 and are attenuated after oocyte specification in controls but that these RNAs persisted in *SETDB1*
178 and *wde* GKD egg chambers (**Figure 2H-O**). Thus, mRNAs that are broadly expressed prior to
179 oocyte specification, become repressed by *SETDB1* and *Wde* in differentiated egg chambers.
180

181 **SETDB1 represses transcription of a subset of targets by increasing H3K9me3 enrichment**

182 To investigate whether *SETDB1*/*Wde*-regulated mRNAs are repressed at the level of
183 transcription, we examined a subset of nascent mRNAs (pre-mRNAs) by qRT-PCR. Indeed, the
184 levels of nascent *RpS19b*, *ord*, *sunn*, *conA* and *blanks* mRNAs were increased in *SETDB1/wde*-
185 GKDs ovaries compared to control WT ovaries (**Figure S3A-B**). These data suggest that
186 transcription of these genes increases upon loss of *SETDB1* or *Wde*.
187

188 To determine if the *SETDB1*-dependent repression of these genes involves changes in H3K9me3,
189 we performed CUT&RUN (Ahmad, 2018; Skene and Henikoff, 2017) on adult WT ovaries
190 enriched for differentiated egg chambers where these genes are repressed (**Figure 2G**). Analysis
191 of CUT&RUN data from adult WT showed enrichment of H3K9me3 marks on previously identified
192 *SETDB1* targets and genes containing heterochromatin such as PHD Finger Protein 7 (*phf7*) and
193 *light* (*lt*) respectively validating our CUT&RUN data (**Figure 3A-B; Figure S3C**) (Devlin et al.,
194 1990; Smolko et al., 2018). As genes in *Drosophila* genome are closely packed, we only analyzed
195 the gene body from 5'UTR to the end of the 3'UTR to unambiguously identify *SETDB1* regulated
196 genes (Schwartz and Cavalli, 2017). We found that 1593 out of 2,316 genes upregulated upon
197 loss of *SETDB1* are enriched for H3K9me3 marks compared to IgG negative control (**Figure 3C**).
198 In addition, we found that 888 genes lose H3K9me3 on their gene bodies upon GKD of *SETDB1*
199 including *RpS19b* and ATP-dependent chromatin assembly factor (*Acf*) (**Figure 3D-F**). The
200 upregulated genes that do not show changes to H3K9me3 marks within the gene body may be
201 regulated by elements outside of the gene body or indirectly. Importantly, taken together, our data
202 suggest that *SETDB1* is required for H3K9me3 enrichment and transcriptional repression of a
203 cohort of early-oogenesis genes in the egg chamber.
204

205 *SETDB1* is required for transposon repression during oogenesis (Andersen et al., 2017; Rangan
206 et al., 2011), and the upregulation of transposons can affect gene expression (Sienski et al., 2012;
207 Upadhyay et al., 2016). However, we found that the upregulation of genes in the differentiated
208 stages that we observed upon depletion of *SETDB1* was not due to the secondary effect of
209 transposon upregulation as the expression of *RpS19b* reporter was not altered in germline
210 depleted of *aubergine* (*aub*), a critical component of the piRNA pathway (**Figure S3D-F**) (Chen
211 et al., 2007; Czech et al., 2018; Malone et al., 2009; Wang et al., 2015). Nor, did *aub* depletion
212 cause mid-oogenesis death as we observed in *SETDB1* and *wde* GKDs (**Figure S3D-F**) (Chen
213 et al., 2007; Wilson et al., 1996). Overall, our data suggest that loss of *SETDB1* derepresses a
214 subset of genes during late oogenesis via decreased H3K9me3, independent of transposon
215 dysregulation.
216

217 **SETDB1 is required for the expression of NPC components**

218 GO term analysis of downregulated targets of *SETDB1/wde* GKD included genes that regulate
219 transposition, consistent with the previously described role of *SETDB1/Wde* in the piRNA pathway
220 and those that regulate proper oocyte development, consistent with the previously described

221 phenotype (**Figure 4A**) (Andersen et al., 2017; Clough et al., 2007; Koch et al., 2009; Rangan et
222 al., 2011).

223

224 Unexpectedly, we observed that genes involved in nucleocytoplasmic transport were
225 downregulated in *SETDB1/wde*-GKD ovaries as compared to controls (**Figure 4A**).
226 Nucleocytoplasmic transport is mediated by Nucleoporin complexes (NPCs), which span the
227 nuclear membrane and consist of a cytoplasmic ring, a central scaffold spanning the nuclear
228 envelope, and a nuclear ring and basket (**Figure 4B**) (M. Capelson et al., 2010; Doucet and
229 Hetzer, 2010; Gozalo and Capelson, 2016). Beyond regulating nucleocytoplasmic transport,
230 NPCs also regulate gene transcription, for instance by anchoring and maintaining
231 heterochromatic domains (Capelson and Hetzer, 2009; Hou and Corces, 2010; Iglesias et al.,
232 2020; Sarma and Willis, 2012; Sood and Brickner, 2014). We found that GKD of *SETDB1/wde* in
233 the germline resulted in downregulation of 18 out of ~30 nucleoporins (Nups) that make up the
234 Nucleoporin complex in *Drosophila* (**Figure 4C**), including a germline enriched *Nup154* that is
235 critical for oogenesis (Colozza et al., 2011; Gigliotti et al., 1998; Grimaldi et al., 2007). The Nups
236 that were downregulated upon depletion of *SETDB1* and *wde* were not isolated to one specific
237 NPC subcomplex (**Figure 4B-C**).

238

239 We found that nascent mRNAs corresponding to the *SETDB1/Wde* targets *Nup154*, *Nup205* and
240 *Nup107* were downregulated in *SETDB1/wde*-GKD ovaries, whereas the non-target *Nup62* was
241 unaffected, suggesting that *SETDB1/Wde* promotes transcription of a cohort of Nups (**Figure 4D**).
242 In addition, the levels of a *Nup107*-RFP fusion protein, under endogenous control (Katsani et al.,
243 2008), were significantly reduced in the cysts and egg chambers of *SETDB1*- and *wde*-GKD
244 compared to controls (**Figure S4A-D**).

245

246 To determine if loss of *Nup* expression in *SETDB1/wde*-GKD ovaries resulted in loss of NPC
247 formation, we performed immunofluorescence with an antibody that is known to mark NPCs in
248 *Drosophila* (Maya Capelson et al., 2010; Davis and Blobel, 1987; Hampel et al., 2019; Kuhn et
249 al., 2019). We found that NPC levels were reduced in the egg chambers of *SETDB1/wde*-GKD
250 ovaries compared to controls (**Figure 4E-H**), but the nuclear lamina was unaffected (**Figure S4E-H**),
251 and NPCs in the soma were also unaffected (**Figure 4I**). Thus, *SETDB1/wde* are required for
252 the proper expression of Nups and NPC formation after oocyte specification.

253

254 Heterochromatic genes and piRNA clusters require heterochromatin to promote their transcription
255 (Rangan et al., 2011; Weiler and Wakimoto, 1995). Although we found that *SETDB1* is required
256 for upregulation of Nups, CUT&RUN analysis of H3K9me3 marks revealed that only 3 of the *Nup*
257 genes had any enrichment of H3K9me3 (*Mbo*, *Nup188*, *Gp210*). Moreover, among *SETDB1*-
258 regulated Nups, only *Gp210* showed any heterochromatic enrichment (**Supplemental Table 2**).
259 Taken together, we find that *SETDB1* promotes proper expression of Nups by a yet unknown
260 mechanism in the germline.

261

262 **Nucleoporins are required to maintain heterochromatin domains at the nuclear periphery**

263 Our data so far indicate that, in *Drosophila* female germline, heterochromatin formation mediated
264 by *SETDB1* is required for proper NPC formation by promoting proper expression of a subset of

265 *Nups* including *Nup107* and *Nup154* (**Figure 4C**). In yeast, a subset of Nups are part of the
266 heterochromatin proteome and are required to cluster and maintain heterochromatin at the NPC
267 (Iglesias et al., 2020). This subset includes *Nup107* and the yeast homolog of *Nup154*, *Nup155*,
268 which both have reduced expression in *SETDB1/Wde*-GKD compared to controls. We
269 hypothesized that in *Drosophila*, *SETDB1* could promote silencing of early oogenesis genes by
270 promoting heterochromatin formation. This heterochromatin then promotes expression of Nups
271 and NPC formation, which can then help maintain heterochromatin by anchoring it to nuclear
272 periphery and thus promoting silencing of early-oogenesis genes.
273

274 To first determine if heterochromatin and nucleoporins associate in *Drosophila* female germline,
275 we utilized antibody against H3K9me3 to mark heterochromatin and *Nup107*-RFP to mark NPCs
276 in WT ovarioles (Katsani et al., 2008; Rangan et al., 2011). We found that H3K9me3 domains
277 were often at the nuclear periphery, in close proximity with *Nup107*-RFP (**Figure 5A-A2, E**). Next,
278 to determine if loss of *Nups* leads to loss of heterochromatin, we first depleted *Nup154* and probed
279 for heterochromatin formation. We chose *Nup154*, as its loss of function phenotype of *Nup154*
280 has been well described (Gigliotti et al., 1998; Grimaldi et al., 2007). We found that GKD of
281 *Nup154* in the germline, resulted in egg chambers that do not grow and die mid-oogenesis as
282 previously described for *Nup154* mutants (**Figure S5A-B2**) (Gigliotti et al., 1998). In addition,
283 depletion of *Nup154* results in proper translocation of *SETDB1* from the cytoplasm to the nucleus
284 suggesting that transport of *SETDB1* into the nucleus is not grossly affected (**Figure S5C-D1**).
285 By staining for H3K9me3 marks, we found that upon GKD of *Nup154*, heterochromatin domains
286 initially form (**Figure S5E-F2**). However, in the egg chambers of *Nup154* GKD, the colocalization
287 between H3K9me3 domains and *Nup107*-RFP levels at the nuclear periphery were significantly
288 reduced prior to significant reduction of heterochromatin levels (**Figure 5A-D1, Figure S5E-F3**,
289 **I**). GKD of *Nup107* also resulted in egg chambers that do not grow and loss of heterochromatin
290 (**Figure S5E-I**). Thus, *Nups 154* and *107*, which are positively regulated by *SETDB1*, are required
291 for H3K9me3 localization at the nuclear periphery for H3K9me3 maintenance in the female
292 germline.
293

294 **Nups are required for silencing early-oogenesis genes**

295 Based on our findings above that Nups are required to maintain H3K9me3 levels and localization,
296 we hypothesized that they are also required to silence the early-oogenesis RNAs in differentiated
297 egg chambers. To test this hypothesis, we depleted *Nup154* and *Nup107* in the germline of a fly
298 carrying the *RpS19b-GFP* reporter. We found that GKD of these nucleoporins resulted in
299 upregulation of *RpS19b-GFP* phenocopying GKD of *SETDB1/wde* (**Figure 6A-C; Figure S6A-B1, D**). Moreover, germline depletion of *Nup62*, which is within the NPC but not regulated by
300 *SETDB1*, also resulted in upregulation of *RpS19b-GFP* and egg chambers that did not grow
301 (**Figure S6A-D**). This suggests that activity of NPC components and not just the Nups regulated
302 by *SETDB1* are required for silencing *RpS19b-GFP* reporter.
303

305 To determine if Nups are required for silencing other early oogenesis RNAs, we performed RNA-
306 seq, and compared *Nup154* GKD ovaries with young ovaries as a developmental control (**Figure**
307 **S2A**). Using a 1.5-fold cut off (Fold Change (FC) \geq |1.5|) and False discovery rate (FDR) <0.05 ,
308 we found that compared to control, in *Nup154* GKD 2809 genes are upregulated, and 2922 genes

309 are downregulated (**Figure 6D**) (**Supplemental Table 1**). Strikingly, 97% of upregulated genes
310 and 89% of downregulated *SETDB1/Wde* targets overlapped with *Nup154* GKD (**Figure 6E**;
311 **S6E**). *Nup154* was involved in silencing genes that promote oocyte differentiation including
312 synaptonemal complex components *ord*, *sunn* and *conA* as well as *RpS19b* (**Figure 6F; S6F-H**).
313 In addition, GKD of *Nup154* also resulted in upregulation of *blanks* (**Figure 6G**). The levels of
314 *Nup154*-regulated RNAs decreased after the cyst stage, when the oocyte is specified, in contrast
315 to non-targets, which have similar RNA levels at all stages (**Figure 6H; S6I-J**). Thus, *Nup154* is
316 critical for silencing early-oogenic mRNAs in the differentiated egg chambers.
317

318 To determine if *Nup154* is required for H3K9me3 marks at *SETDB1*-regulated gene loci such as
319 *RpS19b*, we carried out CUT& RUN for H3K9me3 in control and *Nup154* GKD. We found that
320 564 out of 622 genes displaying a loss in H3K9me3 in *Nup154* GKD also show the same loss in
321 *SETDB1* GKD including *RpS19b* and *Acf* (**Figure 6I; S6K-L**) (**Supplemental Table 2**). Taken
322 together, we find that Nups are required for silencing and H3K9me3 at a subset of *SETDB1/Wde*-
323 regulated loci.
324

325 **Silencing genes expressed during the early oogenesis stages is required for maintaining 326 oocyte fate**

327 We next asked why loss of *SETDB1*, *wde* and *Nups* results in egg chambers that do not grow
328 and die mid-oogenesis. Egg chambers with oocyte specification or maintenance defects result in
329 death of egg chambers mid-oogenesis (Blatt et al., 2021). To determine if there are oocyte
330 specification or maintenance defects, we stained GKD of *SETDB1*, *wde* and *Nup154* for the
331 oocyte marker Egalitarian (Egl) as well as Vasa and 1B1 (Mach and Lehmann, 1997; Navarro et
332 al., 2004). In the early stages of oogenesis, as in control, GKD of *SETDB1*, *wde* and *Nup154*
333 resulted in one Egl positive cell, suggesting that oocyte is specified (**Figure 7A-E**). While initial
334 Egl localization to oocytes appeared to be normal, we cannot rule out subtle specification defects.
335 However, in the later egg chambers, compared to control ovariole, GKD of *SETDB1*, *wde* and
336 *Nup154* resulted in either mis-localization or diffuse Egl expression suggesting loss of oocyte fate
337 (**Figure 7A-E**). Taken together, these data suggest that *SETDB1*, *Wde* and *Nup154* are required
338 for maintaining the oocyte fate.
339

340 **Discussion**

341 Many maternally contributed mRNAs in oocytes are critical for early development after fertilization
342 (Calvi et al., 1998; Huynh and St Johnston, 2004; Kugler and Lasko, 2009; Navarro et al., 2004;
343 Telfer, 1975). We previously showed that many mRNAs expressed in germ cells and the
344 undifferentiated stages of oogenesis must be selectively degraded and thus excluded from the
345 maternal contribution (Blatt et al., 2021). However, the potential role of transcriptional silencing
346 of germ cell and GSC-enriched genes during oogenesis was unclear. Here, we found that
347 regulated translocation of *SETDB1* into the nucleus during oocyte specification is required to
348 silence germ cell- and early oogenesis-genes in the differentiated egg chambers (**Figure 7F**), and
349 that this process is essential to maintain oocyte fate. Thus, some genes that are expressed in
350 germ cells and some that promote differentiation are transcriptionally silenced at the onset of
351 oocyte specification mediated by a feedback loop between heterochromatin and NPC.
352

353

354 **Regulated heterochromatin formation during oocyte specification promotes germ cell to**
355 **oocyte transition**

356 A large fraction of SETDB1 is cytoplasmic in the undifferentiated stages of the germline. As the
357 oocyte is being specified during differentiation, SETDB1 becomes mostly nuclear (Clough et al.,
358 2014). This translocation of SETDB1 to the nucleus during oocyte specification is mediated by
359 Windei (Wde), the *Drosophila* ortholog of mAM/MCAF1 (Koch et al., 2009; Osumi et al., 2019).
360 Here we find that translocation of SETDB1 to the nucleus during oocyte specification is required
361 to silence germ cell and early-oogenesis genes at the onset of oocyte specification. MCAF1 also
362 regulates the accumulation of SETDB1 in the nucleus in mammalian cells (Tsusaka et al., 2019).
363 In addition, loss of *SETDB1* during mammalian oogenesis results in meiotic defects and infertility
364 (Eymery et al., 2016). These data suggest that regulated heterochromatin formation is conserved
365 to promote proper oogenesis in mammals.

366

367 We discovered that SETDB1 is required to silence two major classes of genes. The first group is
368 involved in GSC differentiation into an oocyte, including critical genes that promote meiosis I. The
369 second group of genes are those that are merely expressed in the germ cells prior to
370 differentiation into an oocyte, but have no specific function in the female germline such as *blanks*
371 (Blatt et al., 2021; Gerbasi et al., 2011). We propose that these genes that are silenced upon
372 oocyte specification are either detrimental to late oogenesis or early embryogenesis. Indeed, it
373 has been shown that overexpression of one such gene *actin 57B* (*act57B*), which is repressed by
374 SETDB1/Wde (**Supplemental Table 1**), is detrimental to oogenesis (Blatt et al., 2021; Duan et
375 al., 2020). Remarkably, some of the mRNAs encoded by genes that are transcriptionally silenced
376 by SETDB1 during this transition are also targeted at the post-transcriptional level for degradation
377 by members of the no go decay pathway such as *blanks* and *Act57B* (Blatt et al., 2021). Thus,
378 our data suggests that the regulation of gene expression during the germ cell to oocyte transition
379 reflects a two-step process: transcriptional silencing dependent on SETDB1, and post-
380 transcriptional degradation of mRNAs to exclude a cohort of germ cell mRNAs from the maternal
381 contribution (Blatt et al., 2021).

382

383 SETDB1 is guided to its target transposons and piRNA clusters mediated by piRNAs (Andersen
384 et al., 2017; Czech et al., 2018; Koch et al., 2009; Osumi et al., 2019). However, our data suggests
385 that the piRNA pathway does not play a part in silencing germ cell and early oogenesis RNAs.
386 We find that loss of *aub* does not result in upregulation of *RpS19b*. This result is consistent with
387 the fact that loss of *aub* and *piwi* in the germline does not result in egg chambers that do not grow
388 (Chen et al., 2007; Wilson et al., 1996). In somatic cells of the gonad, loss of *wde* function
389 eliminates nuclear SETDB1 signal (Osumi et al., 2019; Timms et al., 2016). However, upon
390 depletion of Wde, SETDB1 was still ubiquitinated, a requirement for its nuclear retention (Osumi
391 et al., 2019). This suggests that in absence of Wde, SETDB1 can translocate to the nucleus but
392 cannot find its targets. Osumi et al. (2019) suggested that Wde could mediate SETDB1
393 recruitment to the its targets, leading to H3K9me3 deposition. In mammals, it has been shown
394 that transcriptional factors such as the KRAB domain-containing Zinc finger proteins recruit
395 SETDB1 to the target genes for silencing, but such transcription factors have not been identified
396 in the female gonad (Frietze et al., 2010; Schultz et al., 2002). Thus, SETDB1 targets germ cell

397 and early oogenesis genes for silencing independent of the piRNA pathway but through a yet
398 undetermined mechanism, either through Wde or through yet undetermined transcription factors.
399

400 **Nucleopore complex and heterochromatin are in a feedback loop to promote gene
401 silencing**

402 The NPC not only mediates selective nucleo-cytoplasmic transport of macromolecules but also
403 regulates gene expression by anchoring chromatin domains, including heterochromatin to the
404 nuclear periphery (Maya Capelson et al., 2010; M. Capelson et al., 2010; Holla et al., 2020;
405 Iglesias et al., 2020; Sarma and Willis, 2012). In addition, in yeast, several Nups are also part of
406 the heterochromatin proteome suggesting that NPCs can regulate gene expression by regulating
407 heterochromatin (Brickner et al., 2019; Iglesias et al., 2020). Consistent with these observations,
408 we find that in the female germline of *Drosophila*, NPC and heterochromatin are closely
409 associated. Loss of NPCs due to depletion of individual Nups results in loss of heterochromatin
410 and subsequent upregulation of germ cell and early oogenesis genes resulting in oogenesis
411 defects. The 97% overlap of target genes between *SETDB1*, *wde* and *Nup154* is indicative that
412 Nups are functioning in the same pathway as *SETDB1*. This suggests that in the female germline,
413 not only do NPCs associate with heterochromatin, but that NPCs also play a role in maintaining
414 heterochromatin and gene repression during germ cell to oocyte transition.
415

416 Silencing of early oogenesis genes at the onset of oocyte specification is timed with exit from
417 mitotic cell cycle. *Drosophila* nucleopore complex consists of ~30 different nucleoporins some of
418 which are solubilized during early mitotic cell division (Güttinger et al., 2009; Laurell and Kutay,
419 2011). Nucleoporins are recruited to the chromatin in early anaphase followed by sequential
420 reassembly of the complex (Kiseleva et al., 2001; Kutay et al., 2021). During *Drosophila*
421 oogenesis, in the premeiotic stage, the GSC divides to eventually produce a 16-cell cyst (Huynh
422 and St Johnston, 2004; Koch et al., 1967; Lehmann, 2012; Spradling et al., 2011). Prophase-I of
423 meiosis begins in cysts where the oocyte is also specified (Ables, 2015; Orr-Weaver, 1995). We
424 find that nucleoporins promote silencing of genes that are required for initiation of meiosis I such
425 as *Rbfox1* and synaptonemal complex components *ord*, *sunn* and *conA* once the cysts have
426 stopped dividing and the oocyte is being specified. Taken together, our data suggests a
427 mechanism wherein after the mitotic division of cysts have ceased and meiosis I is initiated, the
428 reassembly of NPC simultaneously promotes silencing of the genes required for the transition
429 from mitotic GSC division to meiotic oocyte specification.
430

431 While NPC association with heterochromatin has been described, remarkably we find that loss of
432 heterochromatin results in attenuated expression of some but not all Nup mRNAs.
433 Heterochromatic genes and piRNA clusters require heterochromatin to promote their transcription
434 (Andersen et al., 2017; Devlin et al., 1990; Rangan et al., 2011). However, by analyzing CUT
435 &RUN data and previously published ChIP data of H3K9me3 marks, we found that only one Nup
436 regulated by *SETDB1* is enriched for H3K9me3 marks. Therefore, *SETDB1* indirectly promotes
437 expression of Nups.
438

439 The number of genes that need to be silenced varies based on cell types and developmental
440 trajectory. How levels of heterochromatin are coupled to their NPC docking sites in the cell was

441 not known. Like heterochromatin levels, the number of NPCs also varies by cell type and during
442 differentiation (McCloskey et al., 2018). How NPC number is regulated during development was
443 not fully understood. Our findings in the female germline suggest an elegant tuning mechanism
444 for heterochromatin and its NPC docking sites. Heterochromatin promotes levels of NPC which
445 then promote heterochromatin maintenance by tethering it to the nuclear periphery. We find that
446 this loop can be developmentally regulated by controlling levels of SETDB1 in the nucleus
447 mediated by conserved protein Wde to promote heterochromatin formation.

448

449 **Acknowledgements:**

450

451 We are grateful to all members of the Rangan laboratory for discussion and comments on the
452 manuscript. We also thank Dr. Thomas Hurd and Dr. Miler Lee for their comments on the
453 manuscript. We would like to thank Sontheimer lab for Blanks antibody, Lehmann lab for Egl
454 antibody, Bloomington Drosophila Stock Center, Vienna Drosophila Resource Center, Transgenic
455 GKD Project (NIH/NIGMS R01-GM084947), The BDGP Gene Disruption Project, and FlyBase for
456 fly stocks and reagents. Furthermore, we would like to thank CFG Facility at the University at
457 Albany (UAlbany) for performing RNA-seq analyses. P.R. is funded by
458 NIH/NIGMS (RO1GM11177 and RO1GM135628). M.A.S is funded by NIH NIGMS R35 138120
459 and A.V. is funded by R01DE030927.

460

461 **Materials and Methods**

462

463 **Fly lines**

464

465 The following RNAi stocks were used in this study; if more than one line is listed, then both were
466 quantitated and the first was shown in the main figure: *SETDB1* RNAi (Perrimon lab, (Rangan et
467 al., 2011)), *Wde* RNAi (Bloomington #33339), *Nup154* RNAi (Bloomington #34710), *Nup93* RNAi
468 (VDRC #V16189), *Nup62* RNAi (Bloomington #35695), *Nup107* RNAi (Bloomington #43189),
469 *Nup205* RNAi (VDRC #V38608).

470

471 The following tagged lines were used in this study: *dSETDB1-HA* (Bontron Lab) (Seum et al.,
472 2007), *RpS19b-GFP* (Buszczak Lab, (McCarthy et al., 2019), mRFP-Nup107 (Bloomington
#35516).

473

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

476 **Reagents for fly husbandry**

477

Flies were grown at 25-29°C and dissected between 0-3 days post-eclosion.

478 Fly food was made using the procedures as previously described (summer/winter mix) and narrow
479 vials (Fisherbrand Drosophila Vials; Fischer Scientific) were filled to approximately 10-12mL
480 (Flora et al., 2018).

481 **Dissection and Immunostaining**

482 Ovaries were dissected and teased apart with mounting needles in cold PBS and kept on ice. All
483 incubation was done with nutation. Samples were fixed for 10 minutes in 5% methanol-free
484 formaldehyde. Ovaries were washed in 0.5 mL PBT (1X PBS, 0.5% Triton X-100, 0.3% BSA) 4
485 times for 5 minutes each. Primary antibodies in PBT were added and incubated at 4°C nutating
486 overnight. Samples were next washed 3 times for 5 minutes each in 0.5 mL PBT, and once in 0.5
487 mL PBT with 2% donkey serum (Sigma) for 15 minutes. Secondary antibodies were added in PBT
488 with 4% donkey serum and incubated at room temperature for 3-4 hours. Samples were washed
489 3 times for 10 minutes each in 0.5 mL of 1X PBST (0.2% Tween 20 in 1x PBS) and incubated in
490 Vectashield with DAPI (Vector Laboratories) for 1 hour before mounting.

491 The following primary antibodies were used: mouse anti-1B1 (1:20; DSHB), Rabbit anti-Vasa
492 (1:1,000; Rangan Lab), Chicken anti-Vasa (1:1,000; Rangan Lab) (Upadhyay et al., 2016), Rabbit
493 anti-GFP (1:2,000; abcam, ab6556), Rabbit anti-H3K9me3 (1:500; Active Motif, AB_2532132),
494 Mouse anti-H3K27me3 (1:500; abcam, ab6002), Rabbit anti-Egl (1:1,000; Lehmann Lab), Mouse
495 anti-NPC (1:2000; BioLegend, AB_2565026) and Rat anti-HA (1:500; Roche, 11 867 423 001).
496 The following secondary antibodies were used: Alexa 488 (Molecular Probes), Cy3 and Cy5
497 (Jackson Labs) were used at a dilution of 1:500.

498 **Fluorescence Imaging**

499 The tissues were visualized, and images were acquired using a Zeiss LSM-710 confocal
500 microscope under 20X, 40X and 63X oil objective with pinhole set to 1 airy unit. All gain, laser
501 power, and other relevant settings were kept constant for any immunostainings being compared.
502 Image processing was done using Fiji and gain adjustment and cropping was performed in
503 Photoshop CC 2019.

504
505 **Colocalization analysis**
506

507 Confocal images of control and Nup154-RNAi mutants labeled for RFP-Nup107, H3K9me3, and
508 DAPI were imported into Bitplane Imaris 9.6.2 for 3D reconstruction and colocalization analysis.
509 Colocalization between RFP-Nup107 and H3K9me3 was calculated on a per egg chamber basis
510 using the Surface-surface coloc function of Imaris and an automatic threshold detection and the
511 surface-to-surface coloc function. The number of colocalized voxels was then normalized to the
512 number of H3K9me3 voxels (Valm et al., 2017).

513 **Egg laying assays**

514 Assays were conducted in vials with 3 control or experimental females under testing and 1 wild
515 type control males. Crosses were set up in triplicate for both control and experimental. All flies
516 were 1-day old post-eclosion upon setting up the experiment. Cages were maintained at 29°C
517 and plates were changed daily for counting. Analyses were performed for 5 consecutive days.
518 Number of eggs laid were counted and averaged. Adult flies eclosed were counted from all the
519 vials and averaged.

520 **RNA isolation**

521 Ovaries from flies were dissected in cold 1x PBS. RNA was isolated using TRIzol (Invitrogen,
522 15596026) (Blatt et al., 2021; McCarthy et al., 2019).

523 RNA was treated with DNase (TURBO DNA-free Kit, Life Technologies, AM1907), and then run
524 on a 1% agarose gel to check integrity of the RNA.

525 **RNA-seq library preparation and analysis**

526 Libraries were prepared using the Biooscientific kit. To generate mRNA enriched libraries, total
527 RNA was treated with poly(A)tail selection beads (Bioo Scientific Corp., NOVA-512991).
528 Manufacturer's instructions of the NEXTflex Rapid Directional RNA-seq Kit (Bioo Scientific Corp.,
529 NOVA-5138-08) were followed, but RNA was fragmented for 13 minutes. Library quality was
530 assessed with a Fragment Analyzer (5200 Fragment Analyzer System, AATI, Ankeny, IA, USA)
531 following manufacturer's instructions. Single-end mRNA sequencing (75 base pair reads) was
532 performed on biological duplicates from each genotype on an Illumina NextSeq500 by the Center
533 for Functional Genomics (CFG).

534 After quality assessment, the sequenced reads were aligned to the *Drosophila melanogaster*
535 genome (UCSCdm6) using HISAT2 (version 2.1.0) with the RefSeq-annotated transcripts as a
536 guide (Kim et al., 2015). Differential gene expression was assayed by DeSeq2, using a false
537 discovery rate (FDR) of 0.05, and genes with 2-fold or higher were considered significant. The
538 raw and unprocessed data for RNA-seq generated during this study are available at Gene
539 Expression Omnibus (GEO) databank under accession number: XXX. GO term enrichment on
540 differentially expressed genes was performed using Panther (Thomas et al., 2006).

541 **Fluorescent *in situ* hybridization**

542 A modified *in situ* hybridization procedure for *Drosophila* ovaries was followed. Probes were
543 designed and generated by LGC Biosearch Technologies using Stellaris® RNA FISH Probe
544 Designer, with specificity to target base pairs of target mRNAs. Ovaries (3 pairs per sample) were
545 dissected in RNase free 1X PBS and fixed in 1 mL of 5% formaldehyde for 10 minutes. The
546 samples were then permeabilized in 1mL of Permeabilization Solution (PBST+1% Triton-X)
547 rotating in RT for 1 hour. Samples were then washed in wash buffer for 5 minutes (10% deionized
548 formamide and 10% 20x SSC in RNase-free water). Ovaries were covered and incubated
549 overnight with 1ul of probe in hybridization solution (10% dextran sulfate, 1 mg/ml yeast tRNA, 2

550 mM RNaseOUT, 0.02 mg/ml BSA, 5x SSC, 10% deionized formamide, and RNase-free water) at
551 30°C. Samples were then washed 2 times in 1 mL wash buffer for 30 minutes and mounted in
552 Vectashield.

553 **CUT&RUN assay**

554 Ovaries from flies were dissected in ice cold 1x PBS and ovarioles were separated by teasing
555 after dissection with mounting needles. PBS was removed and the samples were permeabilized
556 in 1mL of Permeabilization Solution (PBST+1% Triton-X) rotating in RT for 1 hour. Samples were
557 then incubated overnight at 4°C in primary antibody dilutions in freshly prepared BBT+ buffer
558 (PBST + 1% BSA + 0.5 mM Spermidine + 2 mM EDTA + 1 large Roche complete EDTA-free
559 tablets). Primary antibody was replaced with BBT+ buffer and quickly washed twice. Samples
560 were then incubated in ~700 ng/ml of pAG-MNase in BBT+ buffer rotating for 4 hours at 25°C.
561 Samples were then quickly washed twice in wash+ buffer (20 mM HEPES pH7.5 + 150 mM NaCl
562 + 0.1% BSA + 0.5 mM Spermidine + 1 large Roche complete EDTA-free tablets in water).
563 Samples were resuspended in 150 μ l Wash+C (wash+ + 100 mM CaCl₂) and incubated for 45
564 minutes on nutator at 4°C. The cleavage reaction was terminated by addition of 150 μ l StopR
565 (NaCl final 200 mM + EDTA final 20 mM + 100 μ g/mL RNaseA) and incubating the sample at 37°C
566 for 30 minutes. Samples were then centrifuged at 16,000 x g for 5 minutes and 300 μ l of the
567 supernatant was collected for DNA discovery. To the supernatant, 2 μ L 10% SDS and 2.5 μ L of
568 20 mg /mL Proteinase K was added and incubated at 50°C for 2 hours. Half of this was kept as a
569 backup and half was used in bead cleanup. 20 μ L AmpureXP bead slurry and 280 μ L MXP buffer
570 (20% PEG8000 + 2.5 M NaCl + 10 mM MgCl₂ in water) was added to the sample and mixed
571 thoroughly followed by 15 minutes incubation at RT. The beads were separated by magnet and
572 supernatant was discarded. The beads were carefully washed with 80% ethanol for 30 seconds,
573 while on the magnetic stand and air dried for 2 minutes. The beads were then resuspended in 10
574 μ L DNase free water.

575 **DNA seq library preparation and analysis**

576 The samples from CUT&RUN assay were used for library preparation using NEBNext® Ultra™
577 DNA Library Prep Kit for Illumina® (E7645, E7103) and adaptor ligated DNA were prepared
578 without size selection.

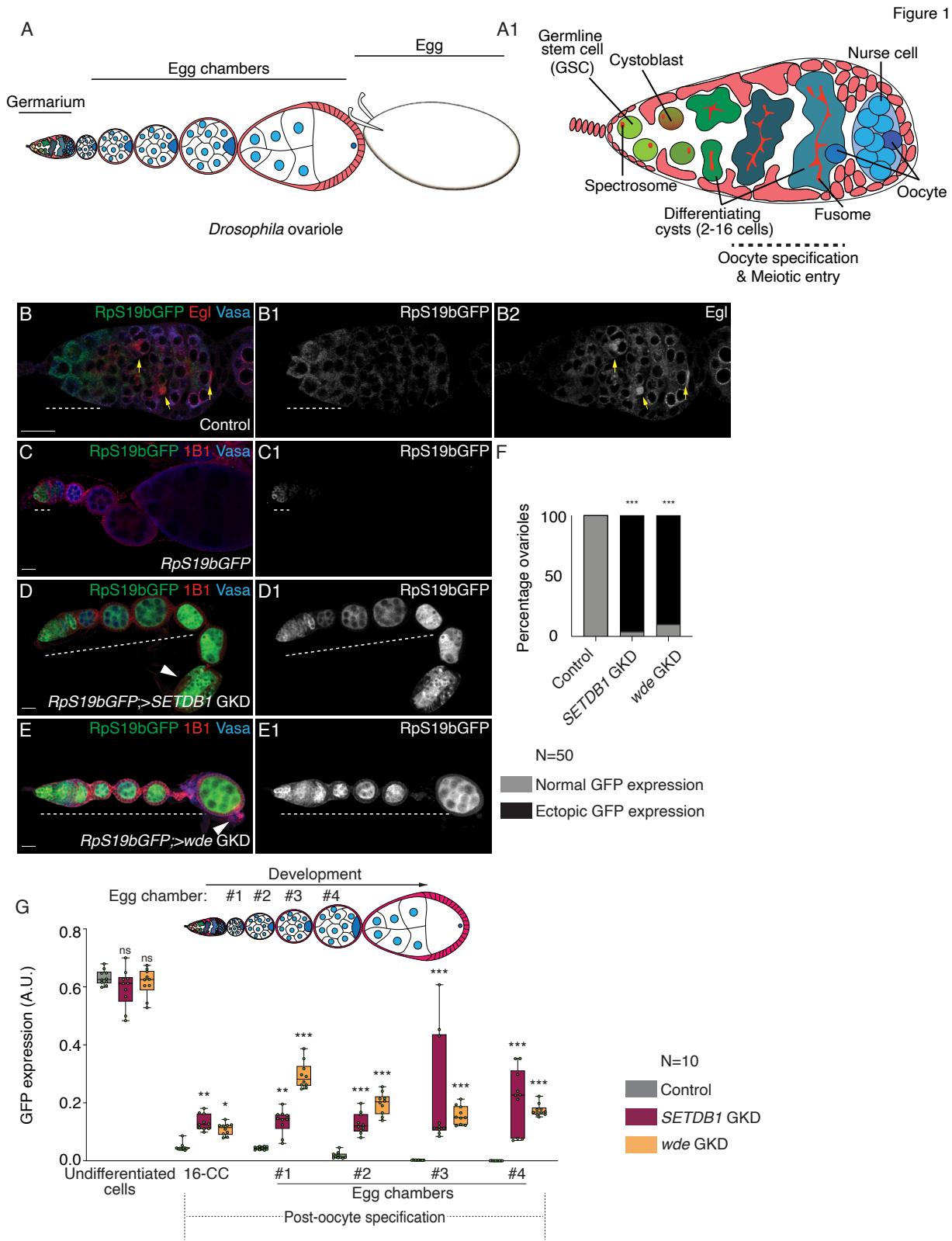
579 **CUT&Run Data Analysis**

580 Cut&Run libraries were sequenced as paired-end 75bp reads on the Illumina NextSeq 500 at the
581 University at Albany Center for Functional Genomics. FASTQ files were aligned to the dm6
582 reference genome using HISAT2 (10.1038/s41587-019-0201-4) (-X 10 -I 1000 –no-spliced-
583 alignment, --no-discordant). Mapping statistics and data will be available from Gene Expression
584 Omnibus. Alignment files were sorted and indexed using samtools and were subsequently used
585 to create bigwig files for visualization with deeptools (–binSize 10)(10.1093/nar/gkw257).
586 Principle component analysis between samples was performed using the multiBigwigSummary
587 and plotPCA modules from deeptools. Only gene bodies were considered and problematic

588 genomic regions (blacklist) were removed from the analysis (10.1038/s41598-019-45839-z). Raw
589 read counts of H3K9me3 enrichment across gene bodies was calculated using the HOMER
590 annotateRepeats function and differential enrichment was calculated using DESeq2 (HOMER
591 PMID:20513432, DESeq2 citation 10.1186/s13059-014-0550-8). H3K9me3 occupied genes are
592 those with differential enrichment of H3K9me3 compared to IgG matched control conditions using
593 DESeq2.

594 **Quantitative Real Time-PCR (qRT-PCR)**

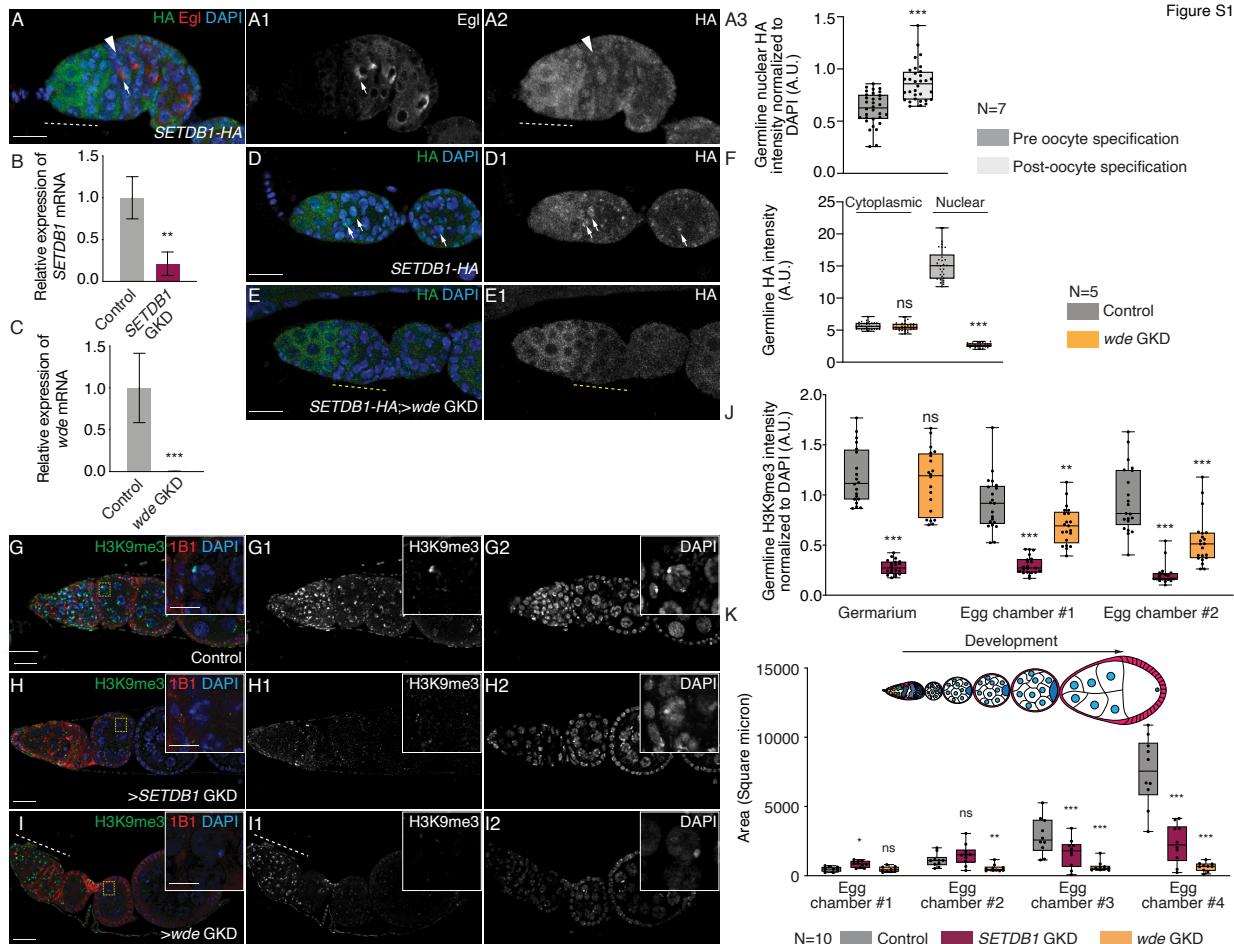
595 1 μ L of cDNA from each genotype was amplified using 5 μ L of SYBR green Master Mix, 0.3 μ L of
596 10 μ M of each reverse and forward primers in a 10 μ L reaction. The thermal cycling conditions
597 consisted of 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C
598 for 60 seconds. The experiments were carried out in technical triplicate and minimum 2 biological
599 replicates for each sample. To calculate fold change in mRNA levels, comparison was done to
600 rp49 mRNA levels which was used as the control gene. Average of the $2^{\Delta\Delta Ct}$ for the biological
601 replicates was calculated. Error bars were plotted using standard error of the ratios and P-value
602 was determined by Students t-test.



603
604

605 **Figure1: SETDB1 and *windei* are required for silencing *RpS19b* reporter during oogenesis**

606
607 (A) A schematic of a *Drosophila* ovariole. The ovariole consists of germarium and egg chambers
608 corresponding to distinct developmental stages. Egg chambers are connected by somatic cells
609 (light red). During development, egg chambers grow and eventually give rise to a mature egg
610 (white).
611
612 (A1) A schematic of a *Drosophila* germarium, where germline stem cells (GSCs; light green) are
613 at close proximity to somatic niche (red). The GSC divides to give rise to daughter cells called
614 cystoblasts (dark green) which turns on a differentiation program. Both GSCs and cystoblasts are
615 marked by spectrosomes (red). Cystoblasts undergo four incomplete mitotic divisions, giving rise
616 to 2-, 4-, 8-, and 16-cell cysts (green), marked by fusomes (red). During the cyst stages germ
617 cells progress through meiotic cell cycle (prophase I). Upon 16-cell cyst formation, a single cell is
618 specified as the oocyte (dark blue) while the other 15 cells become nurse cells (light blue).
619
620 (B-B2) Confocal images of a germarium of a fly carrying *RpS19b-GFP* reporter transgene stained
621 for GFP (green, right grayscale), Egl (red, right grayscale) and Vasa (blue). GFP is expressed in
622 the single cells undifferentiated stages and early cyst stages (white dashed line), while Egl is
623 expressed in the differentiated cysts and localized to the specified oocyte (yellow arrows).
624
625 (C-E) Ovariole of control *RpS19b-GFP* (D-D1), GKD of *SETDB1* (E-E1) and *wde* (F-F1) stained
626 for GFP (green, right grayscale), Vasa (blue) and 1B1 (red). Depletion of these genes resulted in
627 characteristic phenotype of egg chambers not growing and mid oogenesis death (white solid
628 arrows). In addition, ectopic expression of *RpS19b-GFP* was observed in the egg chambers
629 (white dashed line).
630
631 (F) Quantification of ectopic *RpS19b-GFP* expression upon GKD of *SETDB1* or *wde* compared
632 to control ovaries (N= 50 ovarioles; 96% in *SETDB1* GKD and 90% in *wde* GKD compared to 0%
633 in control.) Statistical analysis was performed with Fisher's exact test on ectopic GFP expression;
634 *** = p<0.001.
635
636 (G) Arbitrary units (A.U.) quantification of *RpS19b-GFP* expression in the germarium and egg
637 chambers during development upon GKD of *SETDB1* (magenta) or *wde* (orange) compared to
638 control ovaries (black). GFP is expressed higher in single cells in the germarium, decreases in
639 the cyst stages, and then attenuated upon egg chamber formation. In *SETDB1* and *wde* GKD,
640 GFP expression persists in the egg chambers. Statistical analysis was performed with Dunnett's
641 multiple comparisons test; N= 10 ovarioles; ns = p>0.05, *=p<0.05, ** = p<0.01, *** = p<0.001.
642
643
644 Scale bars are 15 micron.
645
646
647
648
649



650

651

652 Supplementary Figure 1: SETDB1/Wde mediated heterochromatin formation is required for 653 silencing *RpS19b* reporter

654

655 (A-A3) Germarium of a fly carrying HA-tagged SETDB1 stained for HA (green, right grayscale),
656 oocyte marker Egl (red, right grayscale) and DAPI (blue). White arrows point at the specified
657 oocyte. SETDB1 translocates from the cytoplasm (white dotted line) to the nucleus concurrent
658 with oocyte specification (white solid arrow). Quantitation of HA level (A3) expressed as a ratio of
659 nuclear SETDB1 to DAPI. Statistical analysis was performed with Welch's t-test; N= 7 germaria;
660 ns = p>0.05, * = p<0.05, ** = p<0.01, *** = p<0.001

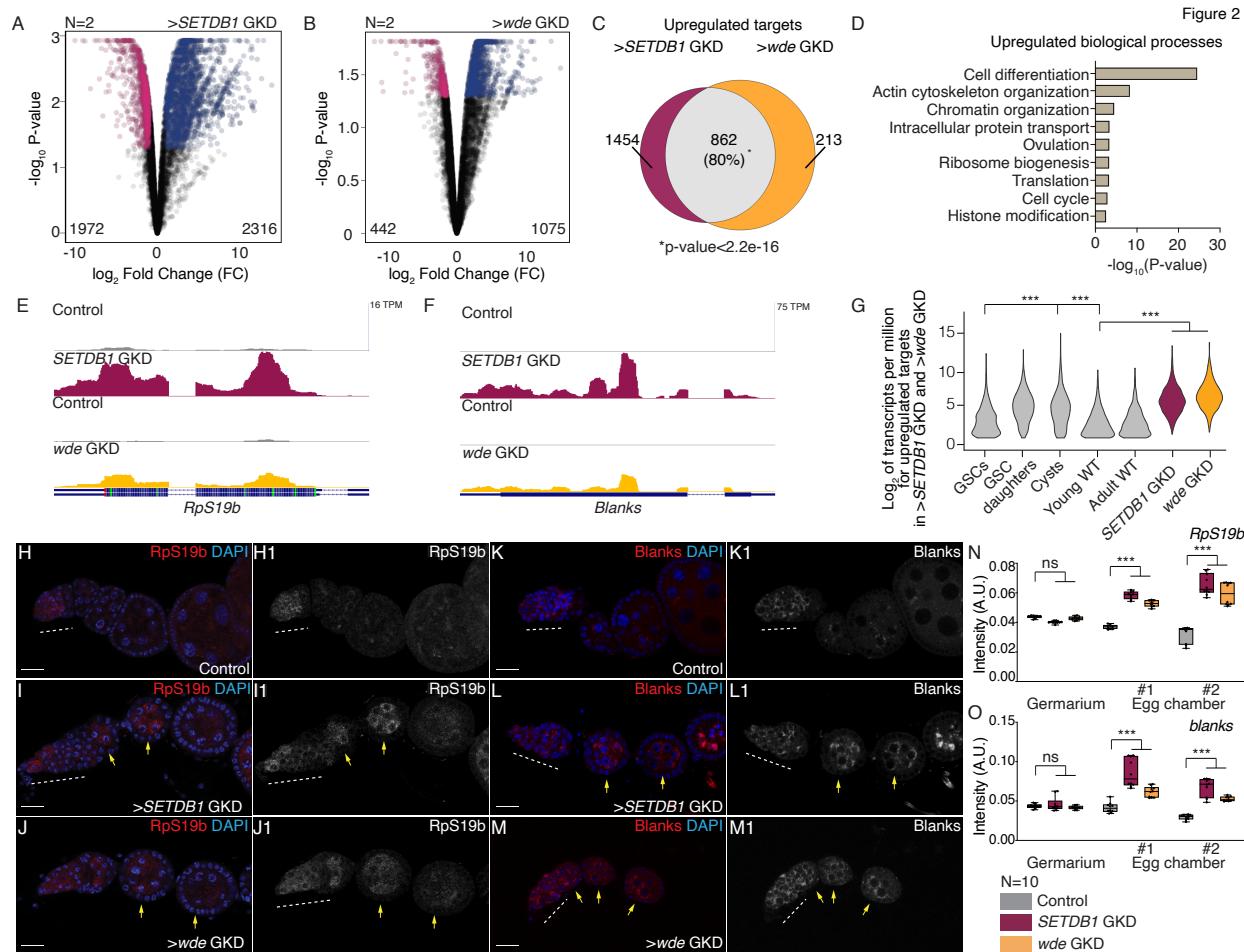
661

662 (B-C) qRT-PCR assaying the mRNA levels of *SETDB1* (B) and *wde* (C) in control and *SETDB1*
663 and *wde* GKD respectively, normalized to control *rp49* mRNA levels and indicating knockdown of
664 these genes (N=3, ** = p<0.01, *** = p<0.001, Error bars are SEM, Student's t-test).

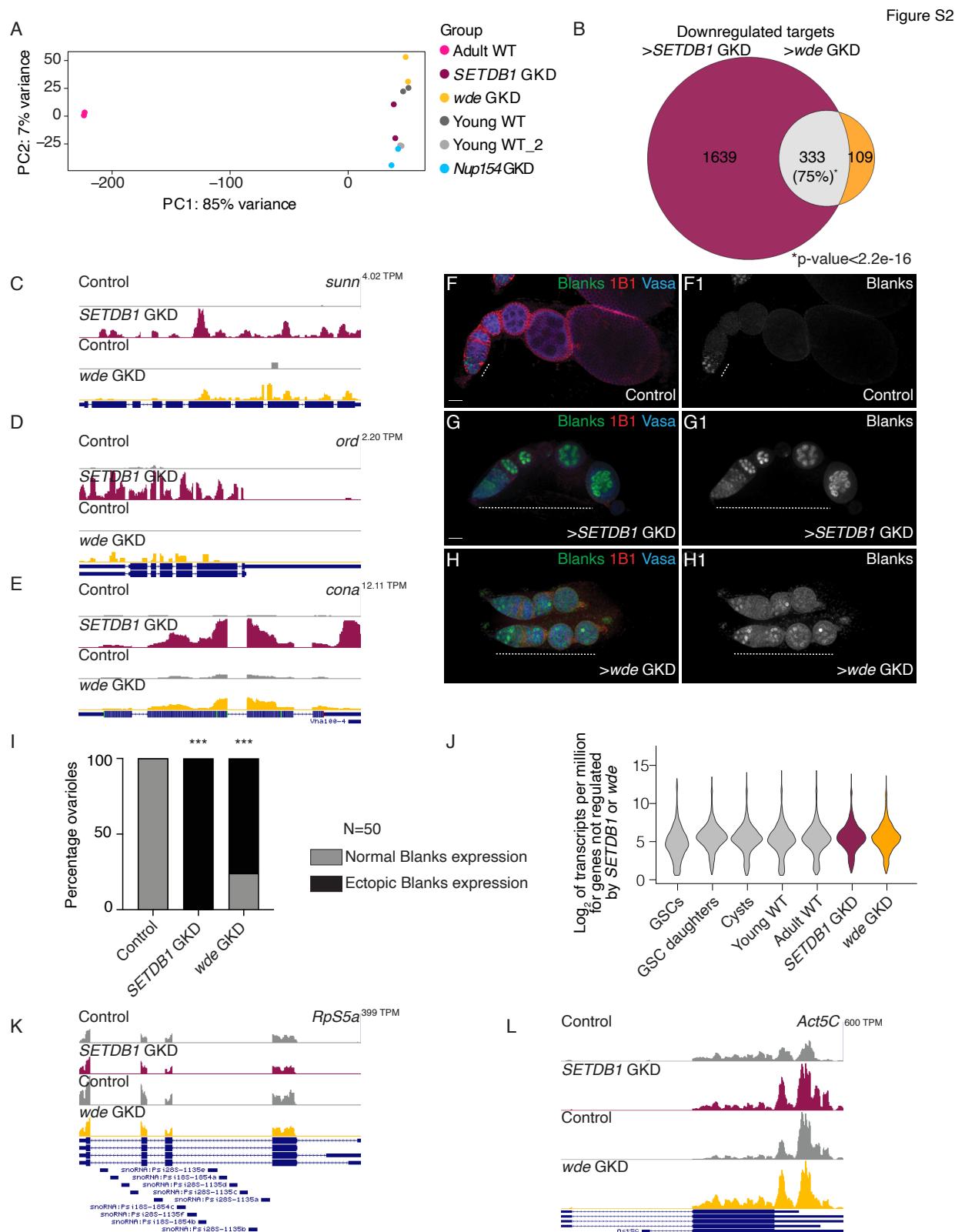
665

666 (D-F) SETDB1-HA ovariole (D-D1) and *SETDB1-HA* ovariole depleted of *wde* (E-E1) stained for
667 HA (green, right grayscale) and Vasa (blue). Yellow arrows point at nuclear HA. GKD of *wde*
668 shows that levels of HA in the nucleus is attenuated (yellow dotted line). (F) Quantification of
669 germline HA levels in the cytoplasm in the undifferentiated stages and in the nucleus of the

670 differentiated stages in the germarium in ovaries depleted of *wde* (orange) compared to control
671 ovaries (gray). Statistical analysis was performed with Welch's t-test; N= 5 ovarioles; ns = p>0.05,
672 * = p<0.05, ** = p<0.01, *** = p<0.001.
673
674 (G-J) Ovariole of control *UAS-Dcr2;nosGAL4* (G-G1), GKD of *SETDB1* (H-H1) or *wde* (I-I1)
675 stained for H3K9me3 (green, right grayscale), DAPI (blue, right grayscale) and 1B1 (red). Nurse
676 cells from egg chamber highlighted by a dashed yellow square represent cells shown in the inset.
677 Control shows H3K9me3 is present throughout oogenesis in the germline. GKD of *SETDB1*
678 shows loss of H3K9me3 in all stages of the germline while depletion of *wde* results in decreased
679 H3K9me3 post-differentiation only in the egg chambers but not in germarium (white dotted line).
680 (J) Quantification of H3K9me3 expression in the germline normalized to DAPI level in ovaries
681 depleted of *SETDB1* (magenta) or *wde* (orange) compared to control ovaries (gray). Statistical
682 analysis was performed with Dunnett's multiple comparisons test; N= 10 ovarioles; ns = p>0.05,
683 * = p<0.05, ** = p<0.01, *** = p<0.001.
684
685 (K) Quantification of area of germarium and egg chambers during development in *SETDB1*
686 (magenta) or *wde* (orange) GKD ovaries compared to control ovaries (gray). Statistical analysis
687 was performed with Dunnett's multiple comparisons test; N= 10 ovarioles; ns = p>0.05, * = p<0.05,
688 ** = p<0.01, *** = p<0.001.
689
690 Scale bars are 15 micron and for main images and scale bar for insets is 4 micron.
691
692
693

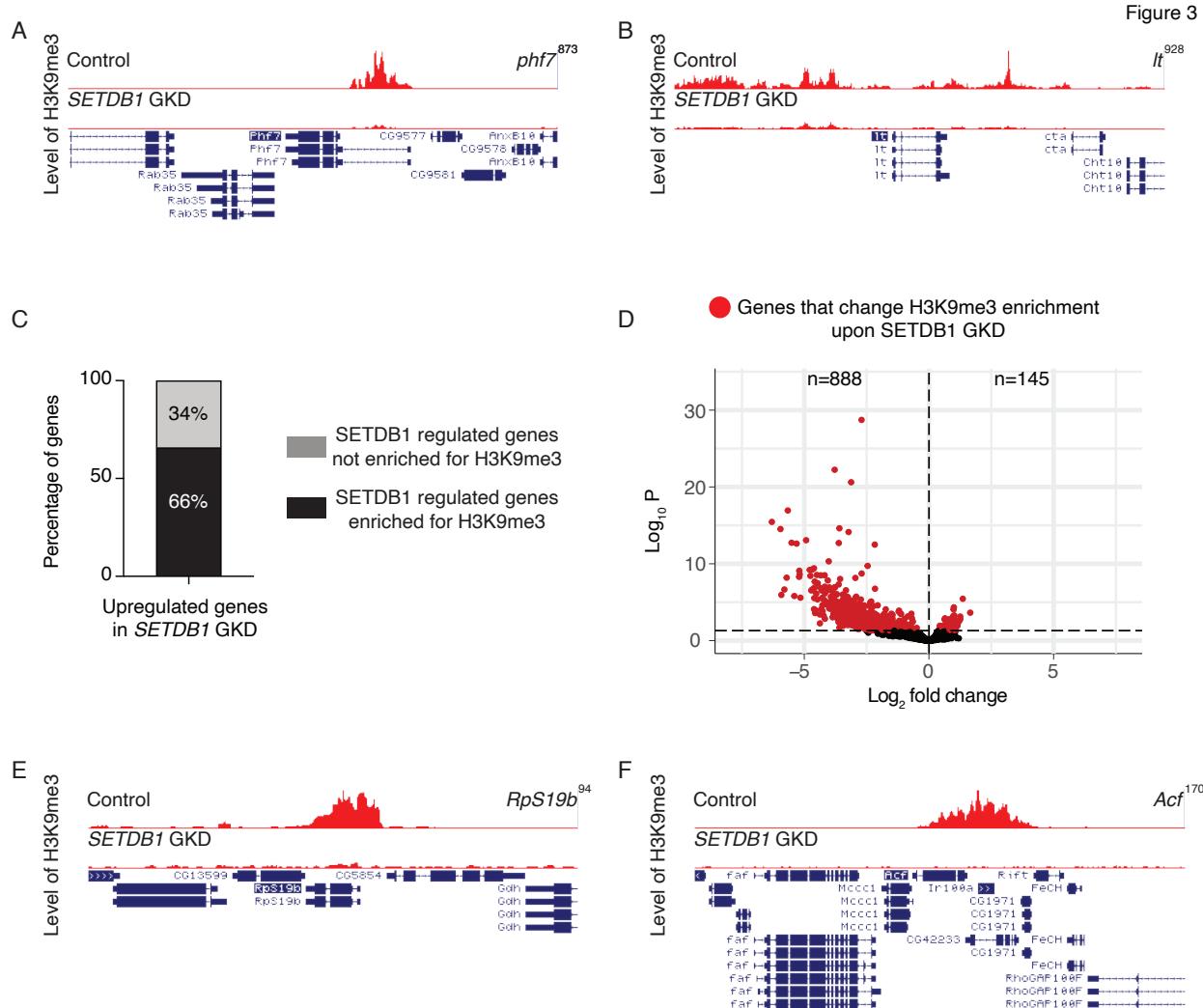


713 (E-F) RNA-seq track showing that *RpS19b* and *blanks* is upregulated upon GKD of *SETDB1* and
714 *wde*.
715
716 (G) Violin plot of mRNA levels of the 862 shared upregulated targets in ovaries enriched for GSCs,
717 cystoblasts, cysts, and whole ovaries, showing that the shared targets between *SETDB1* and *wde*
718 are most highly expressed up to the cyst stages, that then tapers off in whole ovaries. Statistical
719 analysis performed with Hypergeometric test; *** indicates $p < 0.001$.
720
721 (H-J1) Confocal images of germaria probed for *RpS19b* mRNA (red, grayscale) and DAPI (blue)
722 in *UAS-Dcr2;NG4NGT* (H-H1) showing *RpS19b* RNA expression restricted to early stages of
723 oogenesis and in GKD of *SETDB1* (I-I1) and *wde* (J-J1) ovarioles showing *RpS19b* mRNA
724 expression is expanded to egg chambers.
725
726 (K-M1) Confocal images of germaria probed for *blanks* mRNA (red, grayscale) and DAPI (blue)
727 in *UAS-Dcr2;NG4NGT* (K-K1) showing *blanks* mRNA expression restricted to early stages of
728 oogenesis and in GKD of *SETDB1* (L-L1) and *wde* (M-M1) ovarioles where *blanks* mRNA
729 expression is expanded to egg chambers.
730
731 (N-O) Quantification of fluorescence intensity of *RpS19b* (N) and *blanks* (O) mRNAs in the
732 germarium and egg chambers during development in ovaries depleted of *SETDB1* (magenta) or
733 *wde* (orange) compared to control ovaries (gray). Statistical analysis was performed with
734 Dunnett's multiple comparisons test; N= 10 ovarioles; ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** =
735 $p < 0.001$.
736



739 **Supplementary Figure 2: SETDB1/Wde represses a cohort of genes that are broadly**
740 **expressed prior to oocyte specification**

741
742 (A) Principal Component Analysis (PCA) comparing RNA-seq data sets including adult WT, young
743 WT, *SETDB1* GKD and *wde* GKD indicates that the *SETDB1*, *wde* and *Nup154* GKD samples
744 are similar to young WT.
745
746 (B) Venn diagram of downregulated genes from RNA-seq of *SETDB1* and *wde* GKD ovaries
747 compared to *UAS-Dcr2;NG4NGT*. 333 targets are shared between *SETDB1* and *wde* GKD.
748
749 (C-E) RNA-seq track showing that synaptonemal complex members *sunn*, *ord* and *conA* are
750 upregulated upon *SETDB1* and *wde* GKD.
751
752 (F-H1) Confocal images of *UAS-Dcr2;NG4NGT* (C-C1), *SETDB1* (D-D1) and *wde* (E-E1) GKD
753 ovarioles stained for 1B1 (red), Vasa (blue) and Blanks protein (green and grayscale) showing
754 expanded Blanks expression in both *SETDB1* and *wde* GKD egg chambers (arrow).
755
756 (I) Quantification of percentage ovarioles with ectopic *Blanks* expression (black) in *SETDB1* or
757 *wde* GKD ovaries to control ovaries (N= 50 ovarioles; 100% in *SETDB1* GKD and 82% in *wde*
758 GKD compared to 0% in control.) Statistical analysis was performed with Fisher's exact test on
759 ectopic *Blanks* expression; *** = p<0.001.
760
761 (J) Violin plot of mRNA levels of the genes not regulated by *SETDB1* or *Wde* in ovaries enriched
762 for GSCs, cystoblasts, cysts, and whole ovaries, showing that *SETDB1* and *wde* non-targets are
763 not attenuated in the later egg chamber ovaries compared to earlier stages of oogenesis.
764
765 (K-L) RNA-seq track showing that levels of non-targets *RpS5a* and *Act5C* are not affected by
766 *SETDB1* or *wde* GKD.
767



768
769

770 **Figure 3: SETDB1 promotes silencing of early oogenesis genes by regulating levels of**
771 **H3K9me3**

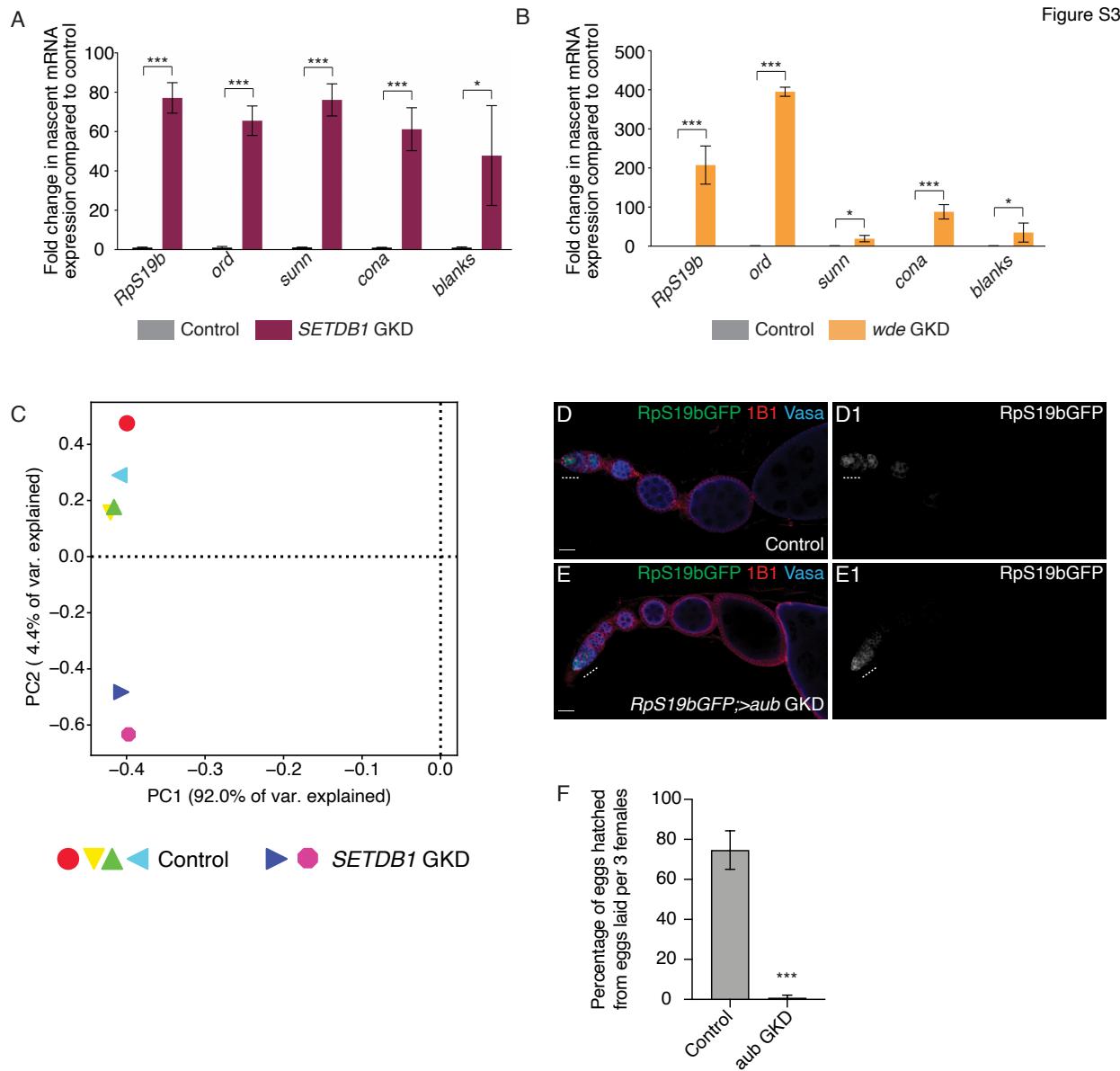
772
773 (A-B) Tracks showing level of H3K9me3 on previously validated and known heterochromatic
774 genes *phf7* and *It* respectively.

775
776 (C) Bar graph showing genes regulated by *SETDB1* that are enriched for H3K9me3 on the gene
777 body. 1593 (black) out of 2316 (gray) genes upregulated upon loss of *SETDB1* are enriched for
778 H3K9me3.

779
780 (D) Volcano plot showing changes in H3K9me3 in *SETDB1* GKD compared to WT. Genes that
781 lose H3K9me3 are shown on the left (red). 888 genes lose H3K9me3 after *SETDB1* GKD.

782
783 (E-F) Tracks showing level of H3K9me3 on target genes. Our data shows loss of H3K9me3 on
784 *SETDB1* targets *RpS19b* and *Acf* respectively (E-F) suggesting they are directly regulated by
785 *SETDB1*.

787
788



789
790

**791 Supplementary Figure 3: SETDB1/Wde transcriptionally silences expression of a subset of
792 early oogenesis genes**

793
794
795
796

(A) qRT-PCR assaying the pre-mRNA levels of *SETDB1*-regulated target genes, including *RpS19b*, *ord*, *sunn*, *cona* and *blanks* in control and GKD of *SETDB1* shows that these genes are upregulated (n=3, *** indicates p<0.001, Error bars are SEM, Student's t-Test).

797
798
799

(B) qRT-PCR assaying the pre-mRNA levels of *Wde*-regulated target genes, including *RpS19b*, *ord*, *sunn*, *cona* and *blanks* in control and GKD of *Wde* shows that pre-mRNA levels of these

800 genes are upregulated (n=3, * = p ≤ 0.05, ** = p<0.01, *** = p<0.001, Error bars are SEM,
801 Student's t-Test).

802

803 (C) Principal Component Analysis (PCA) comparing CUT&RUN data sets for control and *SETDB1*
804 GKD.

805

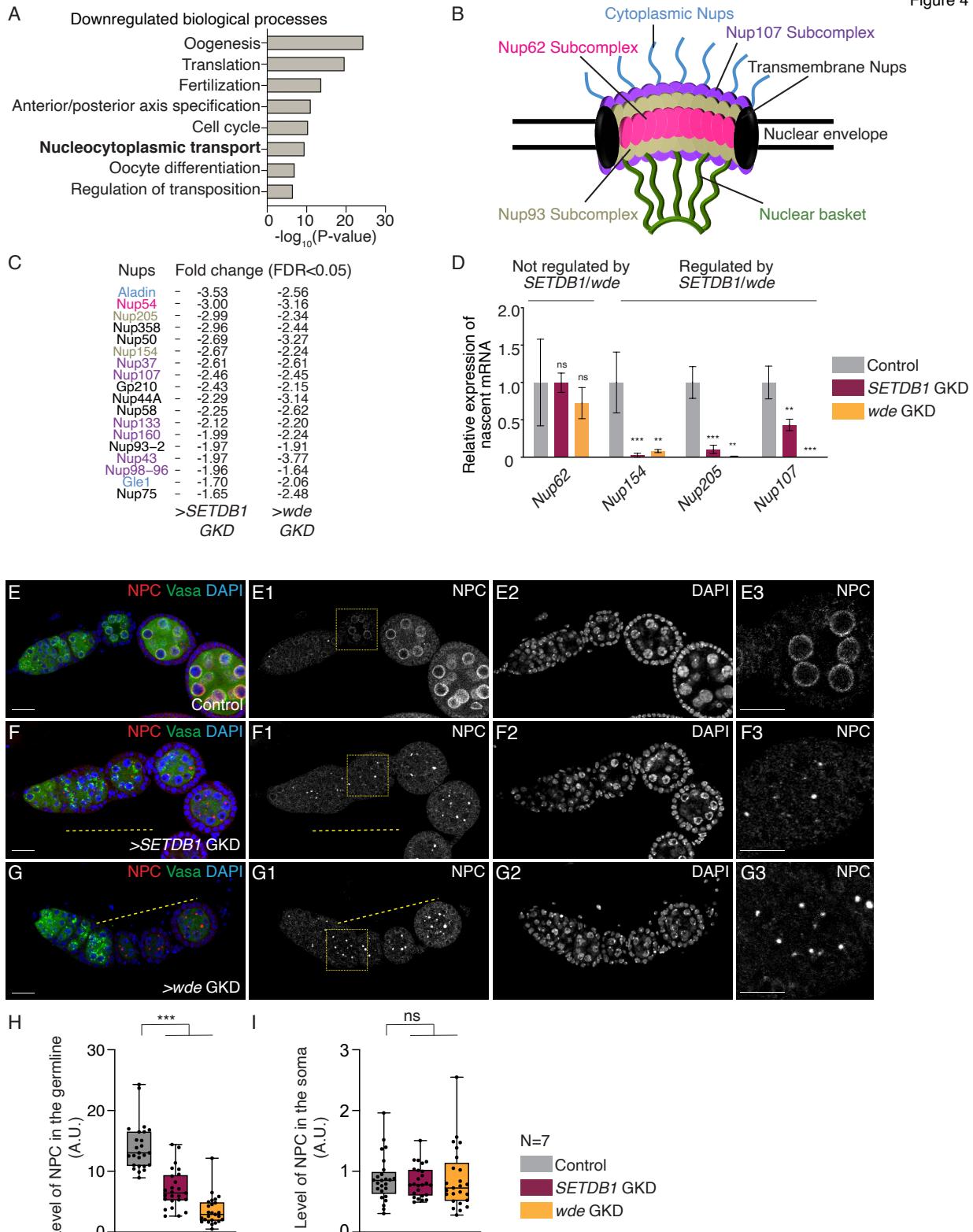
806 (D-E1) Ovariole from control *RpS19b*-GFP (D-D1) and GKD of *aub* (E-E1) stained for GFP (green,
807 right grayscale), Vasa (blue) and 1B1 (red). Depletion of this gene shows normal development of
808 the egg chambers and there was no ectopic expression of *RpS19b*-GFP in the egg chambers
809 suggesting SETDB1-mediated silencing of *RpS19b*-GFP is independent of piRNA pathway.

810

811 (F) Fertility assay of *aub* GKD indicating there was significant decrease in number of adult flies
812 that eclosed from the eggs laid by *aub* GKD flies compared to those from control flies (n=3 trials).
813 *** = p < 0.001, Tukey's post-hoc test after one-way ANOVA.

814

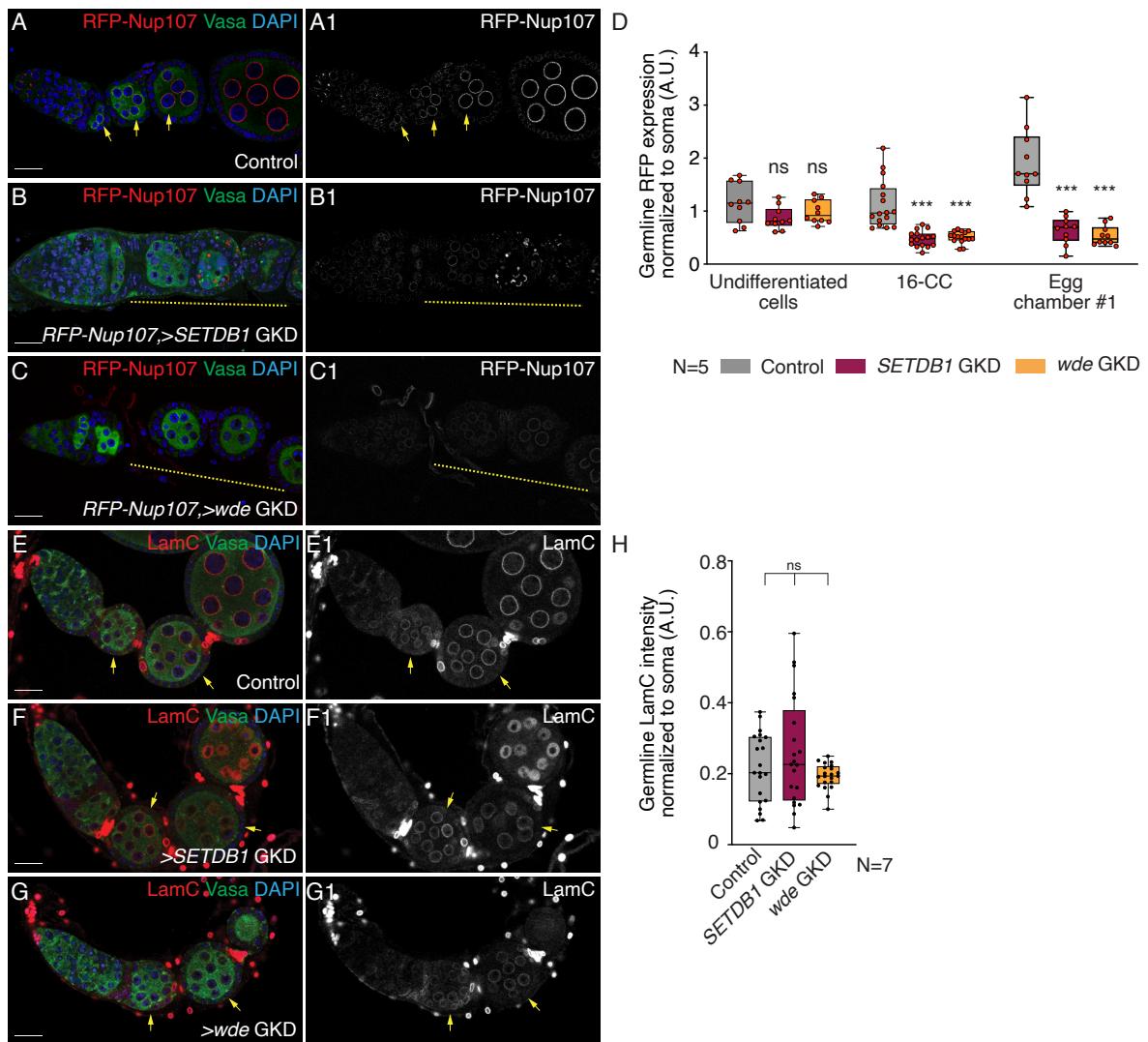
Figure 4



817 **Figure 4: SETDB1/Wde promotes expression of a subset of nucleoporin genes and NPC**
818 **formation**

819
820 (A) The significant biological process GO terms of common downregulated genes in *SETDB1* or
821 *wde* GKD ovaries compared to *UAS-Dcr2;NG4NGT* control (FDR from p-values using a Fisher's
822 exact test), showing nucleocytoplasmic transport as one of the significant processes regulated by
823 *SETDB1/Wde*.
824
825 (B) A schematic of the Nucleopore Complex (NPC) showing cytoplasmic ring, nuclear ring and
826 basket facing nucleoplasm and a central scaffold spanning the nuclear membrane. NPC is
827 composed of several subcomplexes and ~30 nucleoporins (nups).
828
829 (C) Table showing levels of 18 nucleoporin mRNAs that are down regulated 1.5 or more fold in
830 both *SETDB1* or *wde* GKD ovaries compared to *UAS-Dcr2;NG4NGT* control ovaries.
831
832 (D) qRT-PCR assaying the pre-mRNA levels of *SETDB1* and *Wde*-regulated *Nup* genes,
833 including *Nup154*, *Nup205* and *Nup107* are decreased compared to control *UAS-Dcr2;NG4NGT*
834 while levels of non-target *Nup62* pre-mRNA is not affected (control level vs *SETDB1* GKD and
835 *wde* RNA in=3, ** = p<0.01, *** = p<0.001, Error bars are SEM, Student's t-Test).
836
837 (E-G3) Ovariole and egg chamber images of control *UAS-Dcr2;NG4NGT* (E-E3), GKD of *SETDB1*
838 (F-F3) and *wde* (G-G3) stained for NPC (red, grayscale), Vasa (green) and DAPI (blue). NPC
839 staining was done using mab414 antibody. Depletion of *SETDB1* shows reduced expression of
840 NPC in the egg chambers suggesting *SETDB1* regulates expression of several nucleoporins
841 which in turn regulates formation of NPC.
842
843 (H-I) A.U. quantification of NPC level in the germline (H) and soma (I) in *SETDB1* and *wde* GKD
844 ovaries compared to *UAS-Dcr2;NG4NGT* control. Statistical analysis was performed with
845 Dunnett's multiple comparisons test; N= 25 ovariole for germline and 15 for somatic quantitation;
846 ns = p>0.05, * = p ≤ 0.05, ** = p<0.01, *** = p<0.001.
847
848
849

Figure S4



852 **Supplementary Figure 4: SETDB1/Wde promotes NPC formation without affecting Lamin**
853 **C**

854
855 (A-C1) Ovariole of control *RFP-Nup107* A-A1), GKD of *SETDB1* (B-B1) and *wde* (C-C1) stained
856 for RFP (red, right grayscale), Vasa (green) and DAPI (blue). Depletion of *SETDB1* or *wde* shows
857 lower expression of RFP in the egg chambers (yellow line) suggesting *SETDB1/wde* regulates
858 expression of Nup107.

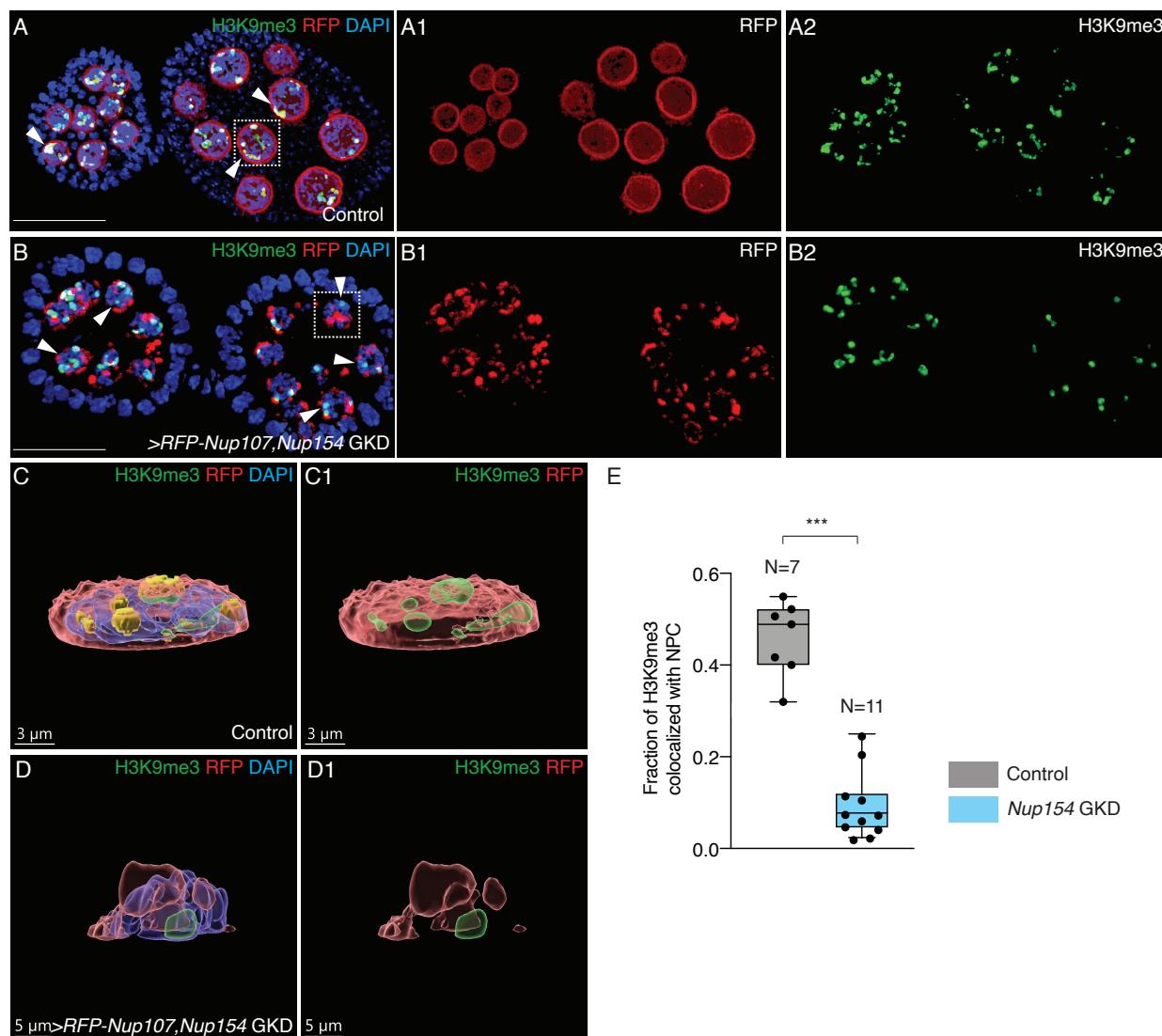
859
860 (D) A.U. quantification of RFP level in the germline normalized to soma in *SETDB1*- and *wde*-
861 GKD ovaries compared to control. Statistical analysis was performed with Dunnett's multiple
862 comparisons test; N= 5 ovariole; ns = p>0.05, * = p ≤ 0.05, ** = p<0.01, *** = p<0.001.

863
864 (E-G1) Ovariole of control *UAS-Dcr2;NG4NGT* (E-E2), GKD of *SETDB1* (F-F2) and *wde* (G-G2)
865 stained for LamC (red, right grayscale), Vasa (green) and DAPI (blue). Depletion of *SETDB1* or
866 *wde* shows similar expression of LamC in the egg chambers suggesting *SETDB1* or *wde*
867 depletion does not affect expression of LamC.

868
869 (H) A.U. quantification of LamC level in the germline normalized to soma in *SETDB1*- and *wde*-
870 GKD ovaries compared to *UAS-Dcr2;NG4NGT* control. Statistical analysis was performed with
871 Dunnett's multiple comparisons test; N= 7 ovariole; ns = p>0.05.

872
873
874
875
876
877
878

Figure 5



879
880

881 **Figure 5: H3K9me3 heterochromatin colocalizes with NPC component Nup107 at the**
882 **nuclear periphery**

883

884 (A-A2) Egg chambers of control *UAS-Dcr2;NG4NGT* ovariole showing RFP-Nup107 (red, right
885 red channel), H3K9me3 (green, right green channel). Heterochromatin is seen in close
886 association with NPC (white arrows). Colocalized fraction is shown in yellow.

887

888 (B-B2) Egg chambers of *Nup154* GKD ovariole showing significant decrease in the colocalization
889 between RFP-Nup107 (red, right red channel) and H3K9me3 (green, right green
890 channel).

891

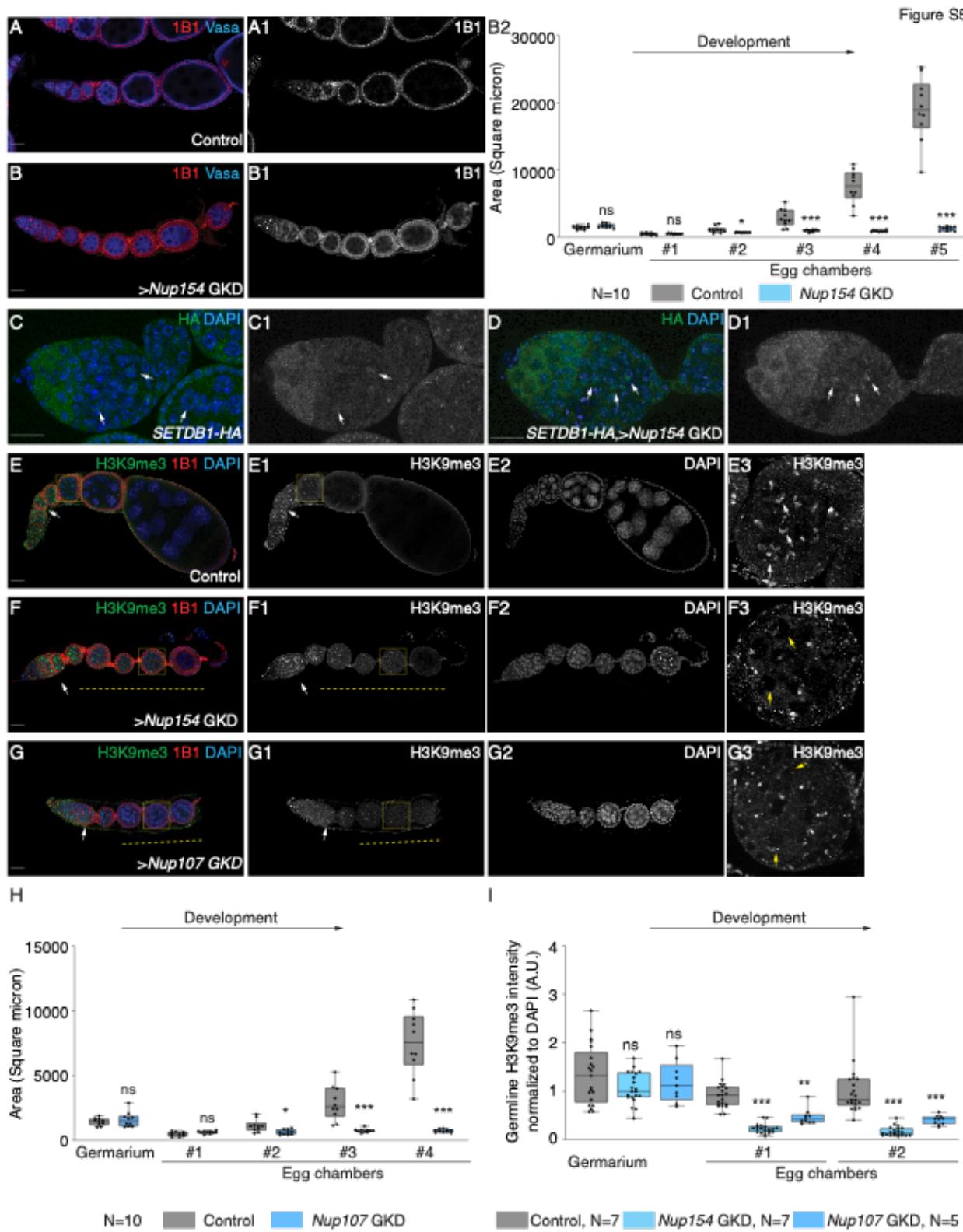
892 (C-C1) 3D reconstruction of a single nuclei (white dotted box in A) from an egg chamber of control
893 *UAS-Dcr2;NG4NGT* ovariole. Yellow channel shows colocalized fraction of RFP-Nup107 (red)

894 and H3K9me3 (green). This shows that H3K9me3 heterochromatin domains (green) are formed
895 at the nuclear periphery and closely associate with Nup107.
896

897 (D-D1) 3D reconstruction of a single nuclei (white dotted box in B) from an egg chamber of
898 *Nup154* GKD showing colocalization (yellow) of H3K9me3 with Nup107. This shows significant
899 reduction in colocalized fraction of H3K9me3 with Nup107.
900

901 (E) Quantification of levels of H3K9me3 that colocalizes with NPC in the germline of control
902 ovarioles (gray) in contrast to *Nup154* GKD ovarioles (blue). Quantitative object based
903 colocalization was measured in Imaris software, *** = p<0.001, one-tailed Students t-Test.
904

905
906
907

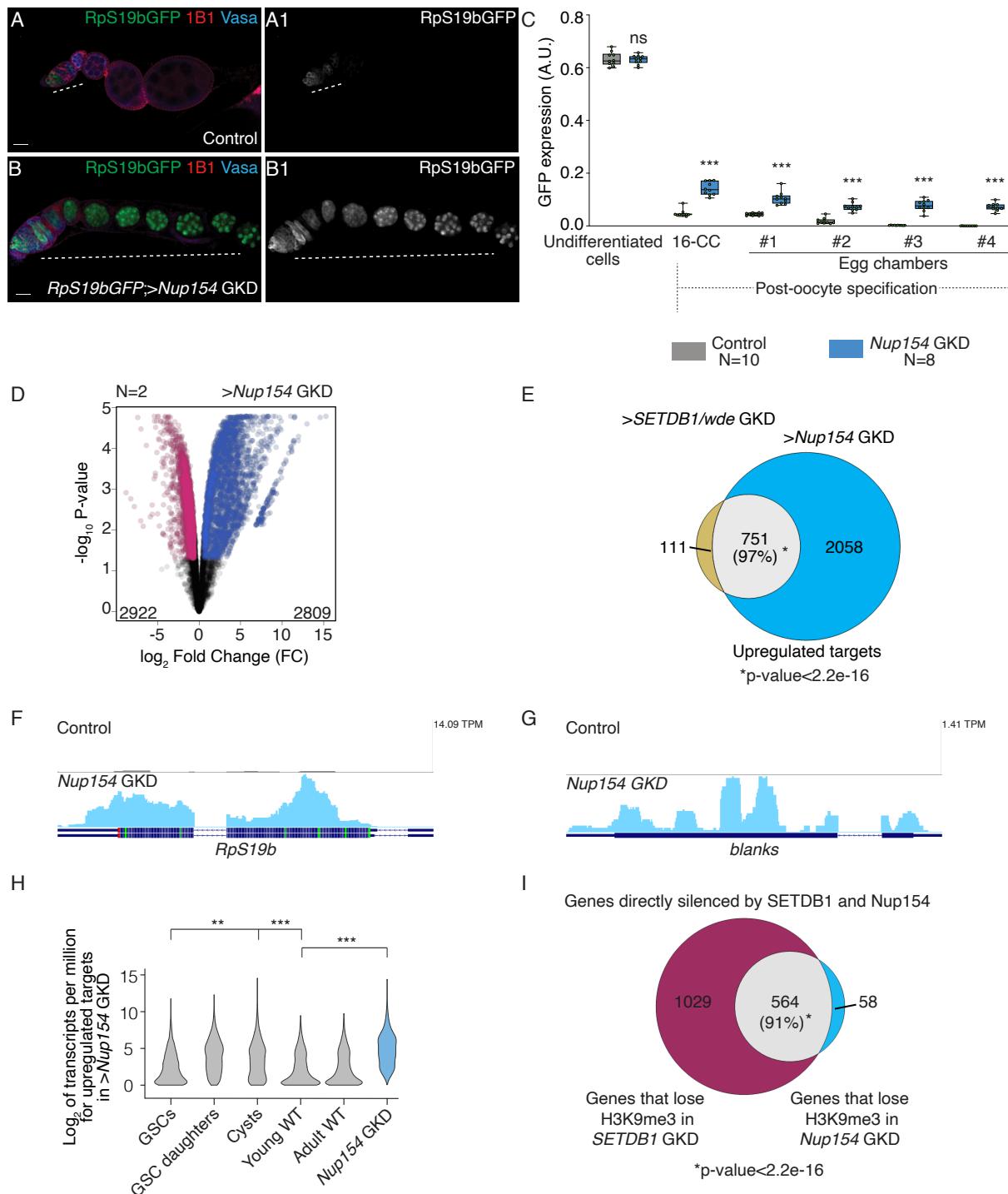


908
909
910

Supplementary Figure 5: NPC is required for maintaining heterochromatin

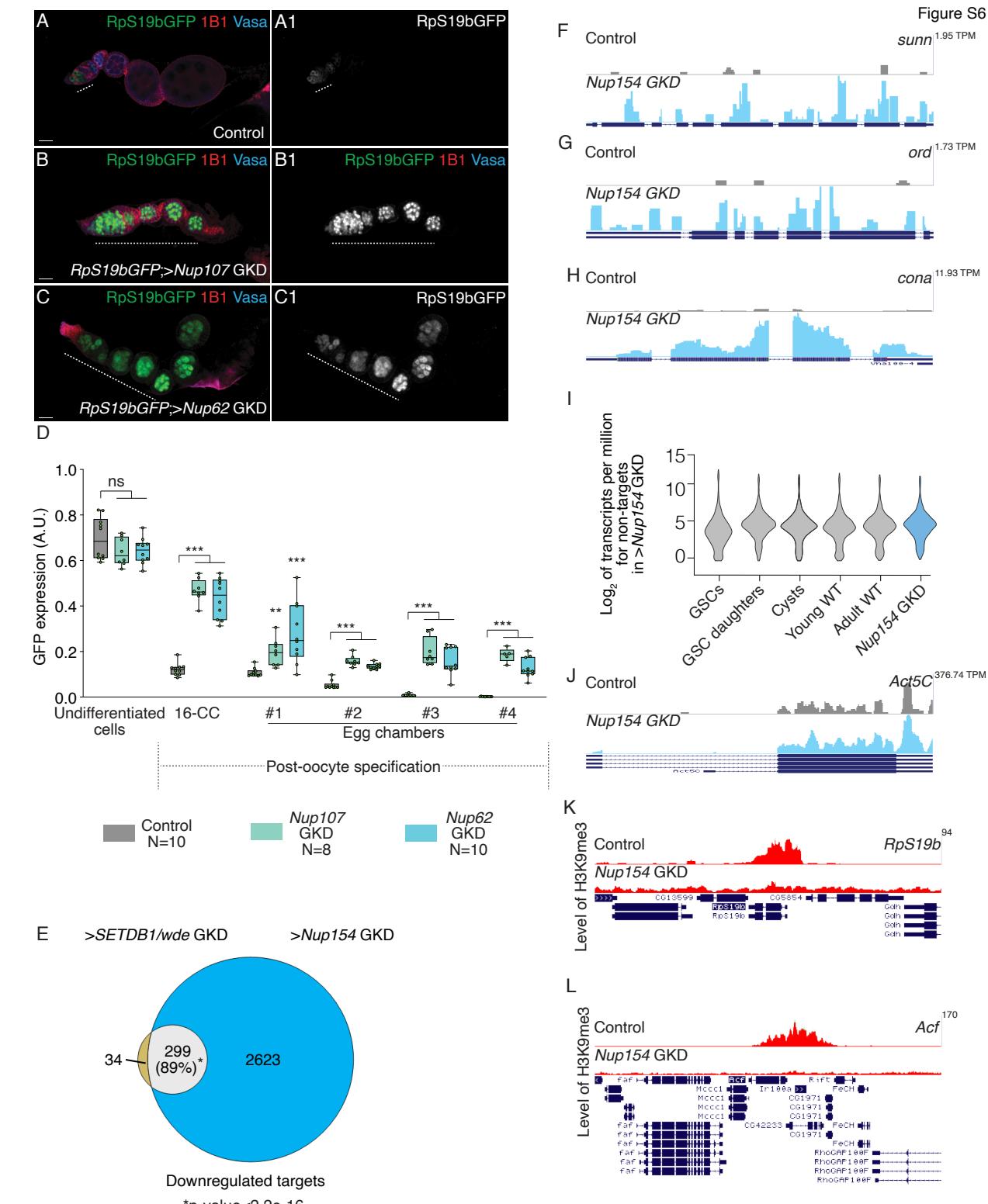
911 (A-B2) Ovariole of control *UAS-Dcr2;NG4NGT* (A-A1) and GKD of *Nup154* (B-B1) stained for 1B1
912 (red, right grayscale) and Vasa (blue). Control shows normal development of egg chambers while
913 *Nup154* GKD shows egg chambers that do not grow. (B2) Quantification of area of gerarium
914 and egg chambers during development in ovaries depleted of *Nup154* (blue) compared to control
915 ovaries (gray). Statistical analysis was performed with Student's t-test; N= 10 ovarioles; ns =
916 $p>0.05$, * = $p \leq 0.05$, ** = $p<0.01$, *** = $p<0.001$.
917
918 (C-D) Germaria of flies carrying HA tagged SETDB1 (C-C1) and GKD of *Nup154* (D-D1) stained
919 for HA (green, right grayscale) and Vasa (blue). White arrows point at nuclear HA. Depletion of
920 germline *Nup154* shows HA is present in the nucleus.
921
922 (E-G3) Ovariole and egg chamber of control *UAS-Dcr2;NG4NGT* (E-E3), GKD of *Nup154* (F-F3)
923 and *Nup107* (G-G3) stained for H3K9me3 (green, right grayscale), DAPI (blue, right grayscale)
924 and 1B1 (red). Control shows H3K9me3 expression throughout oogenesis in the germline.
925 Depletion *Nup154* and *Nup107* results in decreased H3K9me3 (yellow dotted line) after
926 differentiation in the egg chambers. Late-stage egg chamber (yellow dotted squares) images
927 show decreased or loss of H3K9me3 in *Nup154* and *Nup107* GKD nurse cells (yellow arrows).
928
929 (H) Quantification of area of gerarium and egg chambers during development in ovaries
930 depleted of *Nup107* (blue) compared to control ovaries (gray). Statistical analysis was performed
931 with Student's t-test; N= 10 ovarioles; ns = $p>0.05$, * = $p \leq 0.05$, ** = $p<0.01$, *** = $p<0.001$.
932
933 (I) Quantification of H3K9me3 levels in the germline normalized to DAPI level in ovaries depleted
934 of *Nup154* (blue) and *Nup107* (blue) compared to control ovaries (gray). Statistical analysis was
935 performed with Dunnett's multiple comparisons test; ns = $p>0.05$, * = $p<0.05$, ** = $p<0.01$, *** =
936 $p<0.001$.
937
938
939

Figure 6



942 **Figure 6: Nup154 is required for silencing a cohort of genes expressed during early**
943 **oogenesis**

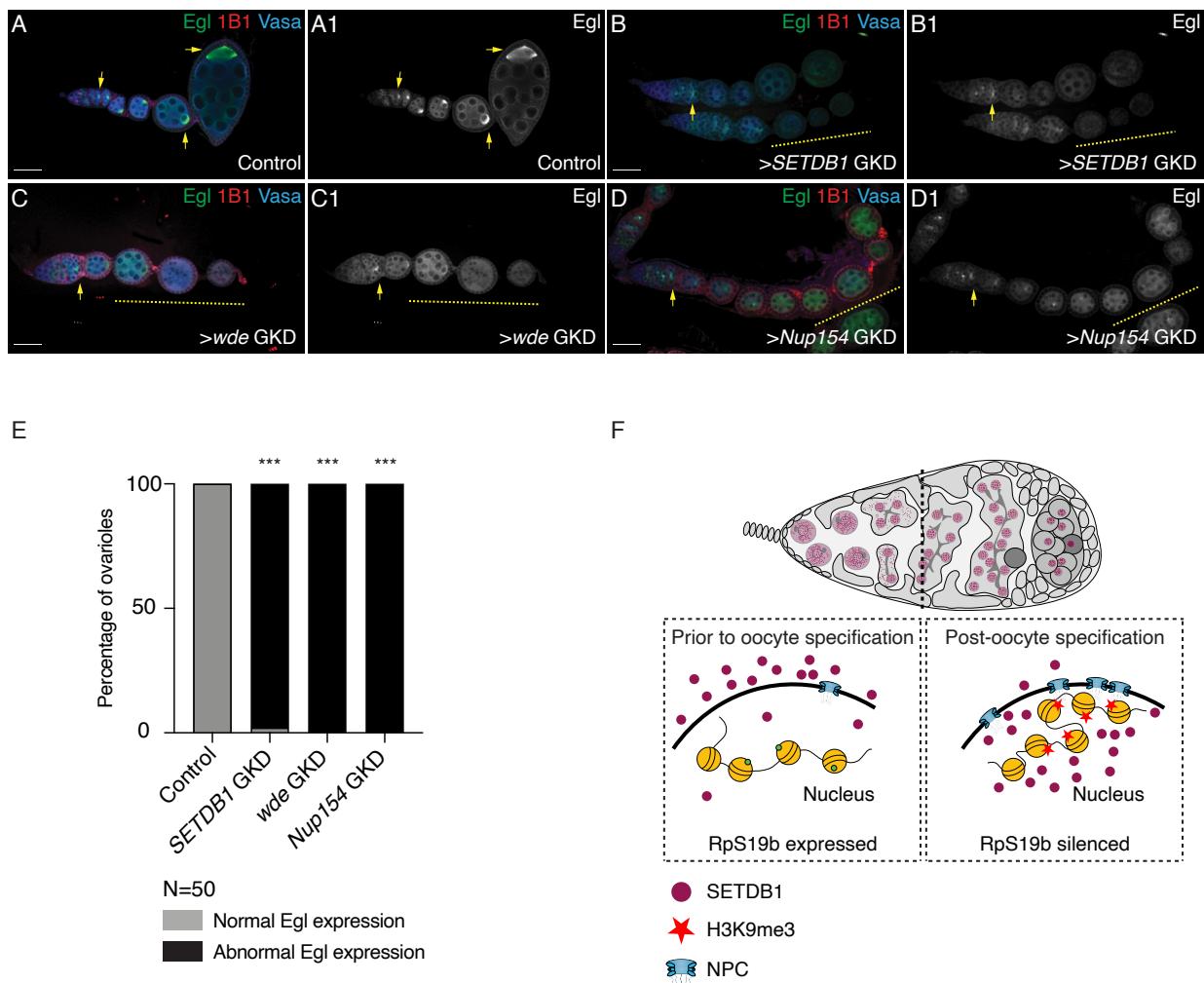
944
945 (A-B1) Ovariole of control *RpS19b-GFP* (A-A1), GKD of *Nup154* (B-B1) stained for GFP (green,
946 right grayscale), Vasa (blue) and 1B1 (red). Depletion of *Nup154* shows characteristic phenotype
947 where the egg chambers did not grow and there was ectopic expression of *RpS19b-GFP* in the
948 egg chambers (white dashed line).
949
950 (C) Arbitrary units (A.U.) quantification of *RpS19b-GFP* expression in the germarium and egg
951 chambers during development upon GKD of *Nup154* (blue) compared to control ovaries (gray).
952 GFP is expressed higher in single cells in the germarium, decreases in the cyst stages, and then
953 attenuated upon egg chamber formation in control. In *Nup154* GKD, GFP expression persists in
954 the egg chambers. Statistical analysis was performed with Dunnett's multiple comparisons test;
955 N= 10 and 8 ovarioles for control and *Nup154* GKD respectively; ns = p>0.05, * = p<0.05, ** =
956 p<0.01, *** = p<0.001.
957
958 (D) Volcano plots of $-\log_{10}P$ -value vs. Log₂Fold Change (FC) of mRNAs that show changes in
959 *Nup154* GKD compared to *UAS-Dcr2;NG4NGT* control ovaries. Pink dots represent significantly
960 downregulated transcripts and blue dots represent significantly upregulated transcripts in *Nup154*
961 GKD ovaries compared with control ovaries (FDR = False Discovery Rate < 0.05 and 1.5-fold or
962 higher change were considered significant).
963
964 (E) Venn diagram of upregulated overlapping genes from RNA-seq of *SETDB1* and *wde* and
965 genes from *Nup154* germline depleted ovaries compared to *UAS-Dcr2;NG4NGT*. 751
966 upregulated targets are shared between *SETDB1*, *wde* and *Nup154* GKD, suggesting that
967 *Nup154* and *SETDB1* function in co-regulating a specific set of genes.
968
969 (F-G) RNA-seq track showing that *RpS19b* (F) and *blanks* (G) are upregulated upon germline
970 depletion of *Nup154*.
971
972 (H) Violin plot of mRNA levels of the 2809 upregulated targets in ovaries enriched for GSCs,
973 cystoblasts, cysts, and whole ovaries, showing that the upregulated targets of *Nup154* are most
974 highly enriched upto the cyst stages, and then tapers off in whole ovaries. Statistical analysis
975 performed with Hypergeometric test; *** indicates p<0.001.
976
977 (I) Venn diagram showing overlapping genes that lose H3K9me3 after depletion of both *SETDB1*
978 and *Nup154* in the germline. 622 genes lose H3K9me3 after *Nup154* GKD out of which 564 genes
979 are also directly silenced by *SETDB1*, suggesting co-regulation of these genes by both *SETDB1*
980 and *Nup154*.
981
982
983
984



Supplementary Figure 6: Nup154 represses the early oogenesis genes by promoting their heterochromatinization

989
990 (A-C1) Ovariole from control *RpS19b*-GFP (A-A1), GKD of *Nup107* (B-B1) and *Nup62* (C-C1)
991 stained for GFP (green, right grayscale), Vasa (blue) and 1B1 (red). Depletion of these *Nups*
992 shows characteristic phenotype where the egg chambers do not grow and there is ectopic
993 expression of *RpS19b*-GFP in the egg chambers (white dashed line).
994
995 (D) A.U. quantification of ectopic *RpS19b*-GFP expression in the germarium and egg chambers
996 with development in ovaries of *Nup107* (teal) and *Nup62* (light blue) GKD compared to control
997 ovaries (gray). Statistical analysis was performed with Dunnett's multiple comparisons test; ns =
998 $p>0.05$, ** = $p<0.01$, *** = $p<0.001$.
999
1000 (E) Venn diagram of down regulated overlapping genes from RNA-seq of *SETDB1* and *wde*
1001 regulated genes with *Nup154* GKD ovaries compared to *UAS-Dcr2;NG4NGT*. 299 down
1002 regulated targets are shared between *SETDB1*, *wde* and *Nup154* GKD, suggesting that *Nup154*
1003 and *SETDB1* function in co-regulating a specific set of genes.
1004
1005 (F-H) RNA-seq track showing that synaptonemal complex members *sunn*, *ord* and *conA* are
1006 upregulated upon germline depletion of *Nup154*.
1007
1008 (I) Violin plot of mRNA levels of the genes not regulated by *Nup154* in ovaries enriched for GSCs,
1009 cystoblasts, cysts, and young and adult whole ovaries, showing that the non-targets of *Nup154*
1010 are not silenced in the ovaries compared to cyst stages and whole ovaries. Statistical analysis
1011 performed with Hypergeometric test; *** indicates $p<0.001$.
1012
1013 (J) RNA-seq track showing that upon germline depletion of *Nup154*, *Act5C* is unaffected.
1014
1015 (K-L) Tracks showing level of H3K9me3 on target genes. H3K9me3 is depleted on *Nup154* targets
1016 *RpS19b* and *Acf* respectively.
1017
1018

Figure 7



1019
1020
1021

1022 **Figure 7: Silencing of early oogenesis genes mediated by SETDB1, Wde and Nup154 is**
1023 **required for maintenance of oocyte fate**

1024
1025 (A-D1) Ovarioles of control *UAS-Dcr2;NG4NGT* (A-A1), GKD of *SETDB1* (B-B1), *wde* (C-C1) and
1026 *Nup154* (D-D2) stained for Egl (green, right grayscale), Vasa (blue) and 1B1 (red). Control shows
1027 proper oocyte specification with one oocyte in each egg chamber. Depletion of *SETDB1*, *wde* and
1028 *Nup154* in the germline results in initial oocyte specification (yellow arrow) which is then lost in
1029 the subsequent egg chambers (yellow dashed line).

1030
1031 (E) Quantification of percentage ovarioles with abnormal/loss of Egl expression (black) in ovaries
1032 depleted of *SETDB1* or *wde* or *Nup154* compared to control ovaries (gray) (N= 50 ovarioles; 98%
1033 in *SETDB1* GKD and 100% in *wde* and *Nup154* GKD compared to 0% in control.) Statistical
1034 analysis was performed with Fisher's exact *** = p<0.001.

1035
1036 (F) A model showing that nuclear translocation of *SETDB1* after differentiation promotes
1037 heterochromatin formation mediated by deposition of H3K9me3 mark. This heterochromatin
1038 promotes increased NPC formation which then helps maintain heterochromatin.

1039
1040

1041 **References**

1042

1043 Ables, E.T., 2015. Drosophila Oocytes as a Model for Understanding Meiosis: An Educational
1044 Primer to Accompany “Corolla Is a Novel Protein That Contributes to the Architecture of
1045 the Synaptonemal Complex of Drosophila.” *Genetics* 199, 17–23.
1046 <https://doi.org/10.1534/genetics.114.167940>

1047 Ahmad, K., 2018. CUT&RUN with Drosophila tissues.
1048 <https://doi.org/10.17504/protocols.io.umfeu3n>

1049 Andersen, P.R., Tirian, L., Vunjak, M., Brennecke, J., 2017. A heterochromatin-dependent
1050 transcription machinery drives piRNA expression. *Nature*.
1051 <https://doi.org/10.1038/nature23482>

1052 Ben-Ami, F., Heller, J., 2005. Spatial and temporal patterns of parthenogenesis and parasitism
1053 in the freshwater snail *Melanoides tuberculata*. *Journal of Evolutionary Biology* 18, 138–
1054 146. <https://doi.org/10.1111/j.1420-9101.2004.00791.x>

1055 Blatt, P., Martin, E.T., Breznak, S.M., Rangan, P., 2020. Post-transcriptional gene regulation
1056 regulates germline stem cell to oocyte transition during Drosophila oogenesis. *Curr Top
1057 Dev Biol* 140, 3–34. <https://doi.org/10.1016/bs.ctdb.2019.10.003>

1058 Blatt, P., Wong-Deyrup, S.W., McCarthy, A., Breznak, S., Hurton, M.D., Upadhyay, M., Bennink,
1059 B., Camacho, J., Lee, M.T., Rangan, P., 2021. RNA degradation is required for the
1060 germ-cell to maternal transition in Drosophila. *Current Biology* 31, 2984–2994.e7.
1061 <https://doi.org/10.1016/j.cub.2021.04.052>

1062 Brickner, D.G., Randise-Hinchliff, C., Lebrun Corbin, M., Liang, J.M., Kim, S., Sump, B., D’Urso,
1063 A., Kim, S.H., Satomura, A., Schmit, H., Coukos, R., Hwang, S., Watson, R., Brickner,
1064 J.H., 2019. The Role of Transcription Factors and Nuclear Pore Proteins in Controlling
1065 the Spatial Organization of the Yeast Genome. *Developmental Cell* 49, 936–947.e4.
1066 <https://doi.org/10.1016/j.devcel.2019.05.023>

1067 Cahoon, C.K., Hawley, R.S., 2016. Regulating the construction and demolition of the
1068 synaptonemal complex, *Nature Structural and Molecular Biology*.
1069 <https://doi.org/10.1038/nsmb.3208>

1070 Calvi, B.R., Lilly, M.A., Spradling, A.C., 1998. Cell cycle control of chorion gene amplification.
1071 *Genes Dev* 12, 734–744. <https://doi.org/10.1101/gad.12.5.734>

1072 Capelson, M., Doucet, C., Hetzer, M.W., 2010. Nuclear pore complexes: guardians of the
1073 nuclear genome. *Cold Spring Harb Symp Quant Biol* 75, 585–597.
1074 <https://doi.org/10.1101/sqb.2010.75.059>

1075 Capelson, M., Hetzer, M.W., 2009. The role of nuclear pores in gene regulation, development
1076 and disease. *EMBO Rep* 10, 697–705. <https://doi.org/10.1038/embor.2009.147>

1077 Capelson, Maya, Liang, Y., Schulte, R., Mair, W., Wagner, U., Hetzer, M.W., 2010. Chromatin-
1078 bound nuclear pore components regulate gene expression in higher eukaryotes. *Cell*
1079 140, 372–383. <https://doi.org/10.1016/j.cell.2009.12.054>

1080 Carreira-Rosario, A., Bhargava, V., Hillebrand, J., Kollipara, R.K., Ramaswami, M., Buszczak,
1081 M., 2016. Repression of Pumilio Protein Expression by Rbfox1 Promotes Germ Cell
1082 Differentiation. *Developmental Cell* 36, 562–571.

1083 Chen, D., McKearin, D., 2003a. Dpp signaling silences bam transcription directly to establish
1084 asymmetric divisions of germline stem cells. *Current biology : CB* 13, 1786–1791.

1085 Chen, D., McKearin, D.M., 2003b. A discrete transcriptional silencer in the bam gene
1086 determines asymmetric division of the Drosophila germline stem cell. *Development*
1087 (Cambridge, England) 130, 1159–1170.

1088 Chen, Y., Pane, A., Schüpbach, T., 2007. Cutoff and Aubergine mutations result in upregulation
1089 of retrotransposons and activation of a checkpoint in the Drosophila germline. *Curr Biol*
1090 17, 637–642. <https://doi.org/10.1016/j.cub.2007.02.027>

1091 Cinalli, R.M., Rangan, P., Lehmann, R., 2008. Germ cells are forever. *Cell* 132, 559–562.
1092 Clough, E., Moon, W., Wang, S., Smith, K., Hazelrigg, T., 2007. Histone methylation is required
1093 for oogenesis in *Drosophila*. *Development* 134, 157–165.
1094 <https://doi.org/10.1242/dev.02698>
1095 Clough, E., Tedeschi, T., Hazelrigg, T., 2014. Epigenetic regulation of oogenesis and germ stem
1096 cell maintenance by the *Drosophila* histone methyltransferase Eggless/dSetDB1.
1097 *Developmental Biology* 388, 181–191. <https://doi.org/10.1016/j.ydbio.2014.01.014>
1098 Colozza, G., Montembault, E., Quérerch'du, E., Riparbelli, M.G., D'Avino, P.P., Callaini, G.,
1099 2011. *Drosophila* nucleoporin Nup154 controls cell viability, proliferation and nuclear
1100 accumulation of Mad transcription factor. *Tissue and Cell* 43, 254–261.
1101 <https://doi.org/10.1016/j.tice.2011.05.001>
1102 Czech, B., Munafò, M., Ciabrelli, F., Eastwood, E.L., Fabry, M.H., Kneuss, E., Hannon, G.J.,
1103 2018. piRNA-Guided Genome Defense: From Biogenesis to Silencing. *Annu Rev Genet*
1104 52, 131–157. <https://doi.org/10.1146/annurev-genet-120417-031441>
1105 Dansereau, D.A., Lasko, P., 2008. The Development of Germline Stem Cells in *Drosophila*.
1106 *Methods Mol Biol* 450, 3–26. https://doi.org/10.1007/978-1-60327-214-8_1
1107 Davis, L.I., Blobel, G., 1987. Nuclear pore complex contains a family of glycoproteins that
1108 includes p62: glycosylation through a previously unidentified cellular pathway. *PNAS* 84,
1109 7552–7556. <https://doi.org/10.1073/pnas.84.21.7552>
1110 Devlin, R.H., Bingham, B., Wakimoto, B.T., 1990. The organization and expression of the light
1111 gene, a heterochromatic gene of *Drosophila melanogaster*. *Genetics* 125, 129–140.
1112 Doucet, C.M., Hetzer, M.W., 2010. Nuclear pore biogenesis into an intact nuclear envelope.
1113 *Chromosoma* 119, 469–477. <https://doi.org/10.1007/s00412-010-0289-2>
1114 Duan, T., Green, N., Tootle, T.L., Geyer, P.K., 2020. Nuclear architecture as an intrinsic
1115 regulator of *Drosophila* female germline stem cell maintenance. *Current Opinion in*
1116 *Insect Science, Development and regulation* 37, 30–38.
1117 <https://doi.org/10.1016/j.cois.2019.11.007>
1118 Eymery, A., Liu, Z., Ozonov, E.A., Stadler, M.B., Peters, A.H.F.M., 2016. The methyltransferase
1119 Setdb1 is essential for meiosis and mitosis in mouse oocytes and early embryos.
1120 *Development* 143, 2767–2779. <https://doi.org/10.1242/dev.132746>
1121 Flora, P., McCarthy, A., Upadhyay, M., Rangan, P., 2017. Role of chromatin modifications in
1122 *Drosophila* germline stem cell differentiation, in: *Results and Problems in Cell*
1123 *Differentiation*. pp. 1–30. https://doi.org/10.1007/978-3-319-44820-6_1
1124 Flora, P., Schowalter, S., Wong-Deyrup, S., DeGennaro, M., Nasrallah, M.A., Rangan, P., 2018.
1125 Transient transcriptional silencing alters the cell cycle to promote germline stem cell
1126 differentiation in *Drosophila*. *Developmental biology* 434, 84–95.
1127 Frietze, S., O'Geen, H., Blahnik, K.R., Jin, V.X., Farnham, P.J., 2010. ZNF274 Recruits the
1128 Histone Methyltransferase SETDB1 to the 3' Ends of ZNF Genes. *PLOS ONE* 5,
1129 e15082. <https://doi.org/10.1371/journal.pone.0015082>
1130 Gerbasi, V.R., Preall, J.B., Golden, D.E., Powell, D.W., Cummins, T.D., Sontheimer, E.J., 2011.
1131 Blanks, a nuclear siRNA/dsRNA-binding complex component, is required for *Drosophila*
1132 spermiogenesis. *PNAS* 108, 3204–3209. <https://doi.org/10.1073/pnas.1009781108>
1133 Gigliotti, S., Callaini, G., Andone, S., Riparbelli, M.G., Pernas-Alonso, R., Hoffmann, G.,
1134 Graziani, F., Malva, C., 1998. Nup154, a new *Drosophila* gene essential for male and
1135 female gametogenesis is related to the nup155 vertebrate nucleoporin gene. *J Cell Biol*
1136 142, 1195–1207. <https://doi.org/10.1083/jcb.142.5.1195>
1137 Gilboa, L., Lehmann, R., 2004. Repression of primordial germ cell differentiation parallels germ
1138 line stem cell maintenance. *Curr Biol* 14, 981–986.
1139 <https://doi.org/10.1016/j.cub.2004.05.049>
1140 Gozalo, A., Capelson, M., 2016. A New Path through the Nuclear Pore. *Cell* 167, 1159–1160.
1141 <https://doi.org/10.1016/j.cell.2016.11.011>

1142 Grimaldi, M.R., Cozzolino, L., Malva, C., Graziani, F., Gigliotti, S., 2007. nup154 genetically
1143 interacts with cup and plays a cell-type-specific function during *Drosophila melanogaster*
1144 egg-chamber development. *Genetics* 175, 1751–1759.
1145 <https://doi.org/10.1534/genetics.106.062844>

1146 Güttinger, S., Laurell, E., Kutay, U., 2009. Orchestrating nuclear envelope disassembly and
1147 reassembly during mitosis. *Nat Rev Mol Cell Biol* 10, 178–191.
1148 <https://doi.org/10.1038/nrm2641>

1149 Hampelz, B., Schwarz, A., Ronchi, P., Bragulat-Teixidor, H., Tischer, C., Gaspar, I., Ephrussi,
1150 A., Schwab, Y., Beck, M., 2019. Nuclear Pores Assemble from Nucleoporin
1151 Condensates During Oogenesis. *Cell* 179, 671-686.e17.
1152 <https://doi.org/10.1016/j.cell.2019.09.022>

1153 Holla, S., Dhakshnamoorthy, J., Folco, H.D., Balachandran, V., Xiao, H., Sun, L., Wheeler, D.,
1154 Zofall, M., Grewal, S.I.S., 2020. Positioning heterochromatin at the nuclear periphery
1155 suppresses histone turnover to promote epigenetic inheritance. *Cell* 180, 150-164.e15.
1156 <https://doi.org/10.1016/j.cell.2019.12.004>

1157 Hou, C., Corces, V.G., 2010. Nups take leave of the nuclear envelope to regulate transcription.
1158 *Cell* 140, 306–308. <https://doi.org/10.1016/j.cell.2010.01.036>

1159 Hughes, S.E., Miller, D.E., Miller, A.L., Hawley, R.S., 2018. Female Meiosis: Synapsis,
1160 Recombination, and Segregation in *Drosophila melanogaster*. *Genetics* 208, 875–908.
1161 <https://doi.org/10.1534/genetics.117.300081>

1162 Huynh, J.-R., St Johnston, D., 2004. The Origin of Asymmetry: Early Polarisation of the
1163 Drosophila Germline Cyst and Oocyte. *Current Biology* 14, R438–R449.
1164 <https://doi.org/10.1016/j.cub.2004.05.040>

1165 Iglesias, N., Paulo, J.A., Tatarakis, A., Wang, X., Edwards, A.L., Bhanu, N.V., Garcia, B.A.,
1166 Haas, W., Gygi, S.P., Moazed, D., 2020. Native Chromatin Proteomics Reveals Role for
1167 Specific Nucleoporins in Heterochromatin Organization and Maintenance. *Mol Cell* 77,
1168 51-66.e8. <https://doi.org/10.1016/j.molcel.2019.10.018>

1169 Jevitt, A., Chatterjee, D., Xie, G., Wang, X.-F., Otwell, T., Huang, Y.-C., Deng, W.-M., 2020. A
1170 single-cell atlas of adult *Drosophila* ovary identifies transcriptional programs and somatic
1171 cell lineage regulating oogenesis. *PLOS Biology* 18, e3000538.
1172 <https://doi.org/10.1371/journal.pbio.3000538>

1173 Katsani, K.R., Karess, R.E., Dostatni, N., Doye, V., 2008. In Vivo Dynamics of *Drosophila*
1174 Nuclear Envelope Components. *MBoC* 19, 3652–3666. <https://doi.org/10.1091/mbc.e07-11-1162>

1175 Kershner, A., Crittenden, S.L., Friend, K., Sorensen, E.B., Porter, D.F., Kimble, J., 2013.
1176 Germline Stem Cells and Their Regulation in the Nematode *Caenorhabditis elegans*, in:
1177 Hime, G., Abud, H. (Eds.), *Transcriptional and Translational Regulation of Stem Cells*,
1178 *Advances in Experimental Medicine and Biology*. Springer Netherlands, Dordrecht, pp.
1179 29–46. https://doi.org/10.1007/978-94-007-6621-1_3

1180 Kiseleva, E., Rutherford, S., Cotter, L.M., Allen, T.D., Goldberg, M.W., 2001. Steps of nuclear
1181 pore complex disassembly and reassembly during mitosis in early *Drosophila* embryos.
1182 *Journal of Cell Science* 114, 3607–3618. <https://doi.org/10.1242/jcs.114.20.3607>

1183 Ko, K., Araúzo-Bravo, M.J., Kim, J., Stehling, M., Schöler, H.R., 2010. Conversion of adult
1184 mouse unipotent germline stem cells into pluripotent stem cells. *Nat Protoc* 5, 921–928.
1185 <https://doi.org/10.1038/nprot.2010.44>

1186 Koch, C.M., Honemann-Capito, M., Egger-Adam, D., Wodarz, A., 2009. Windei, the *Drosophila*
1187 Homolog of mAM/MCAF1, Is an Essential Cofactor of the H3K9 Methyl Transferase
1188 dSETDB1/Eggless in Germ Line Development. *PLoS Genet* 5, e1000644.
1189 <https://doi.org/10.1371/journal.pgen.1000644>

1190 Koch, E.A., Smith, P.A., King, R.C., 1967. The division and differentiation of *Drosophila*
1191 cystocytes. *J. Morphol.* 121, 55–70. <https://doi.org/10.1002/jmor.1051210106>

1193 Kugler, J.-M., Lasko, P., 2009. Localization, anchoring and translational control of oskar,
1194 gurken, bicoid and nanos mRNA during *Drosophila* oogenesis. *Fly (Austin)* 3, 15–28.
1195 <https://doi.org/10.4161/fly.3.1.7751>

1196 Kuhn, T.M., Pascual-Garcia, P., Gozalo, A., Little, S.C., Capelson, M., 2019. Chromatin
1197 targeting of nuclear pore proteins induces chromatin decondensation. *Journal of Cell
1198 Biology* 218, 2945–2961. <https://doi.org/10.1083/jcb.201807139>

1199 Kutay, U., Jühlen, R., Antonin, W., 2021. Mitotic disassembly and reassembly of nuclear pore
1200 complexes. *Trends in Cell Biology*. <https://doi.org/10.1016/j.tcb.2021.06.011>

1201 Lasko, P.F., Ashburner, M., 1988. The product of the *Drosophila* gene *vasa* is very similar to
1202 eukaryotic initiation factor-4A. *Nature* 335, 611–617. <https://doi.org/10.1038/335611a0>

1203 Laurell, E., Kutay, U., 2011. Dismantling the NPC permeability barrier at the onset of mitosis.
1204 *Cell Cycle* 10, 2243–2245. <https://doi.org/10.4161/cc.10.14.16195>

1205 Lehmann, R., 2012. Germline Stem Cells: Origin and Destiny. *Cell Stem Cell* 10, 729–739.
1206 <https://doi.org/10.1016/j.stem.2012.05.016>

1207 Lesch, B.J., Page, D.C., 2012. Genetics of germ cell development. *Nature Reviews Genetics*.
1208 <https://doi.org/10.1038/nrg3294>

1209 Lilly, M.A., Spradling, A.C., 1996. The *Drosophila* endocycle is controlled by Cyclin E and lacks
1210 a checkpoint ensuring S-phase completion. *Genes Dev* 10, 2514–2526.
1211 <https://doi.org/10.1101/gad.10.19.2514>

1212 Mach, J.M., Lehmann, R., 1997. An Egalitarian-BicaudalD complex is essential for oocyte
1213 specification and axis determination in *Drosophila*. *Genes Dev.* 11, 423–435.
1214 <https://doi.org/10.1101/gad.11.4.423>

1215 Malone, C.D., Brennecke, J., Dus, M., Stark, A., McCombie, W.R., Sachidanandam, R.,
1216 Hannon, G.J., 2009. Specialized piRNA Pathways Act in Germline and Somatic Tissues
1217 of the *Drosophila* Ovary. *Cell* 137, 522–535. <https://doi.org/10.1016/j.cell.2009.03.040>

1218 McCarthy, A., Sarkar, K., Martin, E.T., Upadhyay, M., James, J.R., Lin, J.M., Jang, S., Williams,
1219 N.D., Forni, P.E., Buszczak, M., Rangan, P., 2019. MSL3 coordinates a transcriptional
1220 and translational meiotic program in female *Drosophila*. *bioRxiv* 2019.12.18.879874.
1221 <https://doi.org/10.1101/2019.12.18.879874>

1222 McCloskey, A., Ibarra, A., Hetzer, M.W., 2018. Tpr regulates the total number of nuclear pore
1223 complexes per cell nucleus. *Genes Dev.* 32, 1321–1331.
1224 <https://doi.org/10.1101/gad.315523.118>

1225 McKearin, D., Ohlstein, B., 1995. A role for the *Drosophila* bag-of-marbles protein in the
1226 differentiation of cystoblasts from germline stem cells. *Development (Cambridge,
1227 England)* 121, 2937–2947.

1228 McKearin, D.M., Spradling, A.C., 1990. bag-of-marbles: a *Drosophila* gene required to initiate
1229 both male and female gametogenesis. *Genes & development* 4, 2242–2251.

1230 Navarro, C., Puthalakath, H., Adams, J.M., Strasser, A., Lehmann, R., 2004. Egalitarian binds
1231 dynein light chain to establish oocyte polarity and maintain oocyte fate. *Nat Cell Biol* 6,
1232 427–435. <https://doi.org/10.1038/ncb1122>

1233 Orr-Weaver, T.L., 1995. Meiosis in *Drosophila*: seeing is believing. *Proceedings of the National
1234 Academy of Sciences of the United States of America* 92, 10443–10449.

1235 Osumi, K., Sato, K., Murano, K., Siomi, H., Siomi, M.C., 2019. Essential roles of Windei and
1236 nuclear monoubiquitination of Eggless/SETDB1 in transposon silencing. *EMBO Rep* 20,
1237 e48296. <https://doi.org/10.15252/embr.201948296>

1238 Page, S.L., Hawley, R.S., 2001. c(3)G encodes a *Drosophila* synaptonemal complex protein.
1239 *Genes & development* 15, 3130–3143.

1240 Rangan, P., Malone, C.D., Navarro, C., Newbold, S.P., Hayes, P.S., Sachidanandam, R.,
1241 Hannon, G.J., Lehmann, R., 2011. piRNA production requires heterochromatin formation
1242 in *Drosophila*. *Curr Biol* 21, 1373–1379. <https://doi.org/10.1016/j.cub.2011.06.057>

1243 Reik, W., Surani, M.A., 2015. Germline and Pluripotent Stem Cells. *Cold Spring Harb Perspect Biol* 7, a019422. <https://doi.org/10.1101/cshperspect.a019422>

1244 Riparbelli, M.G., Gottardo, M., Callaini, G., 2017. Parthenogenesis in Insects: The Centriole Renaissance. *Results Probl Cell Differ* 63, 435–479. https://doi.org/10.1007/978-3-319-60855-6_19

1245 Sarma, N.J., Willis, K., 2012. The new nucleoporin: regulator of transcriptional repression and beyond. *Nucleus* 3, 508–515. <https://doi.org/10.4161/nucl.22427>

1246 Schultz, D.C., Ayyanathan, K., Negorev, D., Maul, G.G., Rauscher, F.J., 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 16, 919–932. <https://doi.org/10.1101/gad.973302>

1247 Schwartz, Y.B., Cavalli, G., 2017. Three-Dimensional Genome Organization and Function in *Drosophila*. *Genetics* 205, 5–24. <https://doi.org/10.1534/genetics.115.185132>

1248 Seum, C., Reo, E., Peng, H., Rauscher, F.J., Spierer, P., Bontron, S., 2007. *Drosophila SETDB1* Is Required for Chromosome 4 Silencing. *PLoS Genet* 3, e76. <https://doi.org/10.1371/journal.pgen.0030076>

1249 Seydoux, G., Braun, R.E., 2006. Pathway to totipotency: lessons from germ cells. *Cell* 127, 891–904. <https://doi.org/10.1016/j.cell.2006.11.016>

1250 Shapiro-Kulnane, L., Smolko, A.E., Salz, H.K., 2015. Maintenance of *Drosophila* germline stem cell sexual identity in oogenesis and tumorigenesis. *Development* 142, 1073–1082. <https://doi.org/10.1242/dev.116590>

1251 Sienski, G., Dönertas, D., Brennecke, J., 2012. Transcriptional Silencing of Transposons by Piwi and Maelstrom and Its Impact on Chromatin State and Gene Expression. *Cell* 151, 964–980. <https://doi.org/10.1016/j.cell.2012.10.040>

1252 Skene, P.J., Henikoff, S., 2017. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. *eLife* 6, e21856. <https://doi.org/10.7554/eLife.21856>

1253 Smolko, A.E., Shapiro-Kulnane, L., Salz, H.K., 2020. An autoregulatory switch in sex-specific *phf7* transcription causes loss of sexual identity and tumors in the *Drosophila* female germline. *Development* 147. <https://doi.org/10.1242/dev.192856>

1254 Smolko, A.E., Shapiro-Kulnane, L., Salz, H.K., 2018. The H3K9 methyltransferase SETDB1 maintains female identity in *Drosophila* germ cells. *Nat Commun* 9, 4155. <https://doi.org/10.1038/s41467-018-06697-x>

1255 Sood, V., Brickner, J.H., 2014. Nuclear pore interactions with the genome. *Curr Opin Genet Dev* 25, 43–49. <https://doi.org/10.1016/j.gde.2013.11.018>

1256 Spradling, A., Fuller, M.T., Braun, R.E., Yoshida, S., 2011. Germline Stem Cells. *Cold Spring Harb Perspect Biol* 3, a002642. <https://doi.org/10.1101/cshperspect.a002642>

1257 Spradling, Allan C, 1993. Developmental genetics of oogenesis, in: *The Development of Drosophila Melanogaster*.

1258 Spradling, A C, 1993. Germline cysts: communes that work. *Cell* 72, 649–651.

1259 Telfer, W.H., 1975. Development and Physiology of the Oocyte-Nurse Cell Syncytium. *Advances in Insect Physiology*. [https://doi.org/10.1016/S0065-2806\(08\)60164-2](https://doi.org/10.1016/S0065-2806(08)60164-2)

1260 Timms, R.T., Tchaskovnikarova, I.A., Antrobus, R., Dougan, G., Lehner, P.J., 2016. ATF7IP-Mediated Stabilization of the Histone Methyltransferase SETDB1 Is Essential for Heterochromatin Formation by the HUSH Complex. *Cell Rep* 17, 653–659. <https://doi.org/10.1016/j.celrep.2016.09.050>

1261 Tsusaka, T., Shimura, C., Shinkai, Y., 2019. ATF7IP regulates SETDB1 nuclear localization and increases its ubiquitination. *EMBO Rep* 20, e48297. <https://doi.org/10.15252/embr.201948297>

1262 Upadhyay, M., Martino Cortez, Y., Wong-Deyrup, S., Tavares, L., Schowalter, S., Flora, P., Hill, C., Nasrallah, M.A., Chittur, S., Rangan, P., 2016. Transposon Dysregulation Modulates

1293 dWnt4 Signaling to Control Germline Stem Cell Differentiation in Drosophila. PLoS
1294 genetics 12, e1005918.

1295 Valm, A.M., Cohen, S., Legant, W.R., Melunis, J., Hershberg, U., Wait, E., Cohen, A.R.,
1296 Davidson, M.W., Betzig, E., Lippincott-Schwartz, J., 2017. Applying systems-level
1297 spectral imaging and analysis to reveal the organelle interactome. *Nature* 546, 162–167.
1298 <https://doi.org/10.1038/nature22369>

1299 Wang, W., Han, B.W., Tipping, C., Ge, D.T., Zhang, Z., Weng, Z., Zamore, P.D., 2015. Slicing
1300 and Binding by Ago3 or Aub Trigger Piwi-Bound piRNA Production by Distinct
1301 Mechanisms. *Mol Cell* 59, 819–830. <https://doi.org/10.1016/j.molcel.2015.08.007>

1302 Weiler, K.S., Wakimoto, B.T., 1995. Heterochromatin and gene expression in Drosophila. *Annu
1303 Rev Genet* 29, 577–605. <https://doi.org/10.1146/annurev.ge.29.120195.003045>

1304 Wilson, J.E., Connell, J.E., Macdonald, P.M., 1996. aubergine enhances oskar translation in the
1305 Drosophila ovary. *Development* 122, 1631–1639. <https://doi.org/10.1242/dev.122.5.1631>

1306 Xie, T., 2013. Control of germline stem cell self-renewal and differentiation in the Drosophila
1307 ovary: concerted actions of niche signals and intrinsic factors. *Wiley Interdiscip Rev Dev
1308 Biol* 2, 261–273. <https://doi.org/10.1002/wdev.60>

1309 Xie, T., Spradling, A.C., 2000. A niche maintaining germ line stem cells in the Drosophila ovary.
1310 *Science* 290, 328–330. <https://doi.org/10.1126/science.290.5490.328>

1311 Yoon, J., Lee, K.-S., Park, J.S., Yu, K., Paik, S.-G., Kang, Y.-K., 2008. dSETDB1 and
1312 SU(VAR)3–9 Sequentially Function during Germline-Stem Cell Differentiation in
1313 Drosophila melanogaster. *PLOS ONE* 3, e2234.
1314 <https://doi.org/10.1371/journal.pone.0002234>

1315 Yuan, H., Yamashita, Y.M., 2010. Germline stem cells: stems of the next generation. *Curr Opin
1316 Cell Biol* 22, 730–736. <https://doi.org/10.1016/j.ceb.2010.08.013>

1317 Zaccai, M., Lipshitz, H.D., 1996. Differential distributions of two adducin-like protein isoforms in
1318 the Drosophila ovary and early embryo. *Zygote* 4, 159–166.
1319 <https://doi.org/10.1017/s096719940000304x>

1320