Title: The retrotransposon *R2* maintains *Drosophila* ribosomal DNA repeats

One Sentence Summary: The retrotransposon *R2* initiates restoration of ribosomal DNA copies to trans-generationally maintain essential locus.

Authors: Jonathan O. Nelson[1]*, Alyssa Slicko[1,3], and Yukiko M. Yamashita[1,2,3]*

Affiliations: [1]Whitehead Institute for Biomedical Research,
[2]Department of Biology, MIT,
[3]Howard Hughes Medical Institute,
455 Main Street, Cambridge, MA 02142
*Corresponding Authors: jonnels@wi.mit.edu, yukikomy@wi.mit.edu

Abstract: Ribosomal DNA (rDNA) loci contain the hundreds of tandemly repeated copies of ribosomal RNA genes needed to support cellular viability. This repetitiveness makes it highly susceptible to copy number (CN) loss, threatening multi-generational maintenance of rDNA. How this threat is counteracted to avoid extinction of the lineage has remained unclear. Here, we show that the rDNA-specific retrotransposon *R2* is essential for rDNA CN maintenance in the *Drosophila* male germline, despite the perceived disruptive nature of transposable elements. Depletion of *R2* led to defective rDNA CN maintenance, causing a decline in fecundity over generations and eventual extinction. This study reveals that active retrotransposons can provide a benefit to their hosts, contrary to their reputation as genomic parasites, which may contribute to their widespread success throughout taxa.

Main Text:

Ribosomal RNAs (rRNAs) account for 80-90% of all transcripts in eukaryotic cells(*1*). To meet this demand, the ribosomal DNA (rDNA) gene that codes for rRNA is tandemly repeated hundreds of times, comprising rDNA loci on eukaryotic chromosomes. Ironically, this repetitive structure is susceptible to intra-chromatid recombination that causes rDNA copy number (CN) loss **(Fig. 1A)**, which is a major cause of replicative senescence in budding yeast (*2*). Evidence of similar rDNA CN instability has been noted in some tissues from aged dogs and humans(*3*, *4*). Critically, age-associated rDNA CN loss also occurs in the *Drosophila* male germline and is inherited by the next generation(*5*). The essential yet unstable nature of rDNA raises the question as to how the degeneration of rDNA loci over successive generations is prevented to avoid the extinction of the lineage. Intensive studies have revealed that sister chromatid recombination mediates rDNA CN expansion in yeast, thereby maintaining rDNA repeat abundance over generations(*2*). rDNA CN is variable between individuals of most species but maintained within a consistent range throughout the population(*6*), implying that transgenerational dynamic CN changes (loss and restoration) are a common feature of rDNA maintenance. However, the mechanism of

39 transgenerational rDNA CN maintenance in multicellular organisms has remained a mystery.
40 Over 50 years ago, the phenomenon of 'rDNA magnification' in *Drosophila* was first described as
41 the process wherein aberrant rDNA loci bearing minimal rDNA repeats recover to a normal rDNA
42 CN(*7*, *8*). rDNA magnification requires genes involved in homologous recombination-mediated
43 repair, similar to yeast, which can duplicate tandemly repeated elements(*9*). Despite the robust
44 rDNA CN expansion activity, it remained unclear if the mechanisms of rDNA magnification served
45 a physiological function in natural populations. We recently demonstrated that offspring who
46 inherited reduced rDNA CN from old fathers could also recover rDNA CN in their germline(*5*). This
47 recovery depends on the same set of the genes as rDNA magnification, leading us to propose
48 that rDNA magnification is a manifestation of the physiological mechanisms to maintain rDNA CN
49 across generations(*6*). However, the underlying factors responsible for rDNA magnification
50 remains poorly understood.
51
52 Metazoan rDNA genes are frequently inserted by rDNA-specific transposable elements
53 (TEs), such as the retrotransposon *R2* in *Drosophila*. *R2* is found throughout arthropods and *R2*-
54 like elements are widely present across taxa, including Cnidaria, Planaria, nematodes, fish, birds,
55 and reptiles(*10*, *11*). These TEs use their sequence-specific nuclease to mobilize specifically
56 within rDNA loci(*12*), inserting into rDNA genes and likely disrupting 28S rRNA function(*13*) **(Fig.**
57 **S1A)**. Surprisingly, we found that RNAi-mediated knockdown of *R2* in the *Drosophila* male
58 germline (*nos-gal4*-driven expression of RNAi lines, *nos>R2i-1* or *R2i-2,* hereafter) **(Fig. S1A-C)**
59 resulted in premature loss of germline stem cells (GSCs) during aging **(Fig. 1B-F)**. GSCs
60 continuously produce differentiating germ cells to sustain sperm production throughout adulthood
61 and thus are the source of the genome passed to the next generation(*14*). Whereas newly eclosed
62 *R2* RNAi males contained similar numbers of GSCs to controls, GSC number more rapidly
63 declined during aging in *R2* knockdown males compared to controls **(Fig. 1F)**. Given that *R2* is
64 specifically inserted into rDNA, we also examined the effect of *R2* knockdown on rDNA stability.

65 Using highly quantitative droplet digital PCR (ddPCR), we found that RNAi-mediated knockdown
66 of *R2* enhanced rDNA CN loss in the testis during aging **(Fig. 1G-H)**. Interestingly, one of the
67 RNAi constructs (*R2i-2*) suffered rapid rDNA CN loss within the first 10 days of adulthood, but
68 recovered by 20 days of age **(Fig. 1G)**. As GSC number was also initially more drastically affected
69 with this RNAi line **(Fig. 1F)**, we speculate that severe rDNA loss caused by this RNAi construct
70 (*R2i-2*) may select for GSCs that are less sensitive to *R2* RNAi activity. rDNA CN loss in the
71 germline was further confirmed by DNA FISH on the meiotic chromosomes **(Fig S2A-F)**. rDNA
72 CN insufficiency is likely the primary cause of GSC loss in *R2* RNAi animals, because increasing
73 total rDNA CN via introduction of a mini-chromosome harboring an rDNA locus(*15*) suppressed
74 the premature GSC loss caused by *R2* knockdown **(Fig. 1I)**. These results revealed that *R2*
75 contributes to sustaining GSC population during aging through rDNA CN maintenance,
76 uncovering an unanticipated benefit of the *R2* retrotransposon to the host, despite the widely-held
77 view of TEs being genetic parasites.
78
79 Why is *R2* necessary for rDNA copy number maintenance? We found that *R2* is required
80 for rDNA magnification. rDNA magnification is detected as the emergence of offspring with normal
81 cuticle from fathers with abnormal ('bobbed') cuticle caused by insufficient rDNA CN(*7*) **(Fig. 2A)**.
82 *Drosophila melanogaster* rDNA loci reside on the sex chromosomes (X and Y)(*16*), and rDNA
83 magnification almost exclusively occurs to X chromosome rDNA loci harboring the minimal viable
84 amount of rDNA (bb^{Z9}, **Fig. S3A)** when combined with a Y chromosome lacking rDNA (bb^{Z9}/Ybb^{0},
85 'magnifying males' hereafter)(*7*) **(Fig. S3B)**. Importantly, rDNA magnification never occurs in
86 males with a normal Y chromosome containing intact rDNA (bb^{Z9}/Y^{+}, 'non-magnifying males'
87 hereafter)(*8*), indicating the presence of mechanisms to monitor rDNA CN to activate expansion.
88 We found that *R2* knockdown reduces rDNA magnification from 13.73% (control, n = 233) to 0%
89 (*R2i-1*, n = 181, p = 5.6×10^{-7}) and 2.36% (*R2i-2*, n = 127, p =9.9×10^{-4}) **(Fig. 2B)**. Moreover,
90 quantification of rDNA CN by ddPCR revealed that 87.5% of bb^{Z9} chromosomes increased rDNA

91 CN in magnifying males (n = 96, p = $1.8x10^{-4}$), with an average increase of 18.29 rDNA copies
92 across all samples (n = 96, p = $3.1x10^{-12}$) **(Fig. 2C)**. This widespread rDNA CN increase indicates
93 that rDNA magnification broadly increases rDNA CN throughout the germline, despite only
94 13.73% of bb^{Z9} chromosomes recovering enough CN to support normal cuticle development. This
95 rDNA CN increase in magnifying males is also eliminated upon *R2* knockdown **(Fig. 2C)**. These
96 results reveal that *R2* is required for rDNA CN expansion during rDNA magnification. Interestingly,
97 we found that rDNA magnification was blocked only when the *R2* RNAi constructs were expressed
98 by the *nos-gal4* driver in early germ cells (including GSCs), but not when expressed in later germ
99 cells by the *bam-gal4* driver (see Methods) **(Fig. 2B)**. These results indicate that *R2* primarily
100 functions in the earliest stages of germ cells (including GSCs) to support rDNA magnification.
101
102 We further found that *R2* is sufficient for rDNA CN expansion. Ectopic expression of
103 transgenic *R2* in the germline **(Fig. S4A-F)** induced rDNA magnification of the bb^{Z9} locus in non-
104 magnifying males (bb^{Z9}/Y^{+}) **(Fig. 2D-E)**. While rDNA magnification assessed by dorsal cuticle is
105 never detected from control males (bb^{Z9}/Y^{+} without *R2* expression), we found 3.3% of male
106 offspring exhibited magnification (normal cuticle) upon expression of transgenic *R2* **(Fig. 2D,** n =
107 877, p = $3.2x10^{-5}$). Importantly, reversion of the cuticle phenotype was heritable to the subsequent
108 F2 generation throughout our experiments, confirming that CN restoration occurred in the
109 germline **(Fig. S5A-C)**. Quantification of rDNA CN by ddPCR revealed that ectopic
110 overexpression of *R2* in non-magnifying males (bb^{Z9}/Y^{+}) also increases the average rDNA CN at
111 bb^{Z9} rDNA loci among all offspring **(Fig. 2E**, n = 94, p = 0.0256), revealing *R2* is sufficient to
112 induce rDNA CN expansion. Critically, expression of a nuclease dead *R2* transgene (NucDead*R2*)
113 in non-magnifying males **(Fig. S4A-E)** failed to induce rDNA magnification **(Fig. 2D)**, suggesting
114 that the nuclease activity of *R2* is essential for its ability to induce rDNA CN expansion.
115

116 How does *R2*'s nuclease activity contribute to rDNA magnification? In yeast, rDNA CN
117 expansion is initiated by double-stranded breaks (DSBs) at the rDNA intergenic sequence, which
118 induces sister chromatid recombination that results in rDNA gene duplication(*17*). All proposed
119 models of *Drosophila* rDNA CN expansion (the most prominent model being unequal sister
120 chromatid recombination(*18*)) require an initiating DSB at the rDNA locus, **(Fig. 3A; Fig. S6A-B)**.
121 Indeed, artificial introduction of DSBs at rDNA loci by I-CreI endonuclease expression has been
122 reported to induce rDNA magnification(*19*), but the endogenous factor that induces rDNA
123 magnification remained unclear. *R2* is capable of creating DSBs through sequential nicking of
124 opposite DNA strands during retrotransposition(*10*). It has been speculated that DSBs created
125 during *R2* retrotranspostion may be an initiating event of rDNA magnification(*9*), although this
126 possibility has yet to be empirically tested. We confirmed that ectopic overexpression of *R2*, but
127 not NucDead*R2*, indeed induces chromosomal breaks at rDNA loci identified by chromosome
128 spreads **(Fig. S4B-D)**. *R2* overexpression (but not NucDead*R2*) in the germline also increased
129 the frequency of GSCs with DSBs, identified by γH2Av expression **(Fig. S4E)**. Next, we found
130 rDNA magnification is associated with an elevation in DSBs in GSCs: the frequency of γH2Av-
131 positive GSCs is increased in magnifying males (bb^{Z9}/Ybb^{0}) compared to non-magnifying males
132 (bb^{Z9}/Y^{+}) (**Fig. 3B-C, E**; n = 519, p = 8.8×10^{-4}). Strikingly, we observed that knockdown of *R2* in
133 magnifying males reduced the frequency of γH2Av-positive GSCs to levels comparable to non-
134 magnifying males **(Fig. 3D-E;** n = 537, p = 7.1×10^{-4} for *R2i-1*; n = 521, p = 7.9×10^{-4} for *R2i-2***)**,
135 indicating that *R2* is responsible for the DSBs formed in GSCs during rDNA magnification. Taken
136 together, these results suggest that rDNA-specific endonuclease activity of *R2* creates DSBs at
137 the rDNA loci that may in turn induce rDNA CN expansion.
138
139 Given the threat *R2* mobilization poses to the host genome, both by disruption of rRNA
140 function and causing excessive DSB formation(*10*), how is the potential benefit of *R2* to rDNA CN

141 maintenance balanced with the detriment of *R2* retrotransposition? We found *R2* expression in
142 the germline is specifically de-repressed under conditions of reduced rDNA CN, potentially
143 explaining how the conflicting consequences of *R2* expression are resolved. Using RNA
144 fluorescence *in situ* hybridization (RNA FISH) to examine *R2* expression at a single cell resolution,
145 we found that the frequency of GSCs expressing *R2* was significantly increased in magnifying
146 males (bb^{Z9}/Ybb^{0}), whereas non-magnifying males (bb^{Z9}/Y^{+}) rarely expressed *R2* **(Fig. 3F-H**; n =
147 231, p = 1.7×10^{-10}). Moreover, we found that GSCs from aged animals and the sons of old fathers,
148 which inherit reduced rDNA CN(*5*)**,** also exhibited a higher frequency of *R2* expression compared
149 to GSCs from young flies **(Fig. S7A-B, D**; n = 1,247, p = 8.3×10^{-4} for old animals; n = 1,107, p =
150 1.5×10^{-4} for offspring). Importantly, the frequency of *R2* expression among GSCs in the sons of
151 old fathers returned to the basal level after 20 days of age, when rDNA CN was shown to have
152 recovered(*5*) **(Fig. S7C-D**; n = 617, p = 0.036**)**. These results indicate that *R2* expression is
153 dynamically regulated in response to changing rDNA CN. Taken together, we propose that *R2*
154 expression is finely tuned to function when most beneficial to the host while minimizing
155 unnecessary exposure to the harmful effects of transposition.
156
157 Based on the finding that *R2* plays a critical role in maintaining germline rDNA CN, we
158 postulated that *R2* is essential to prevent continuous multi-generational rDNA loss capable of
159 causing the extinction of the lineage. In *C. elegans*, the loss of genome integrity is known to cause
160 gradual loss of fertility, a phenotype known as mortal germline (morg)(*20*). To test whether *R2*-
161 mediated rDNA maintenance is required to maintain fertility through generations, we established
162 multiple independent lines expressing *R2* RNAi in their germline and tracked their fecundity at
163 each generation through the ability of each line to produce sufficient offspring to establish a new
164 generation **(Fig. S8)**. While nearly all control lines survived throughout the duration of the
165 experiments, we found that lines expressing the *R2i-1* RNAi construct failed to consistently
166 produce sufficient progeny, with over half failing by the fourth generation **(Fig. 4A)** (n = 43, p =

167 $3x10^{-6}$), indicating that *R2* is essential for continuity of the germline lineage. Surviving males of
168 extinguishing *R2i-1* lineages had ~20% reduction in rDNA CN compared to control lines (n = 22,
169 p = 0.031) **(Fig. 4B)**. With the *R2i-2* RNAi, the lineage was maintained relatively well, after initial
170 sharp drop **(Fig. 4A)**: Considering that *R2* knockdown by the *R2i-2* construct exhibits only
171 transient rDNA CN decrease at day 10 (**Fig S2A**), we speculate that this RNAi construct quickly
172 selects for the germ cells and lineages insensitive to *R2* knockdown. Taken together, these results
173 suggest that *R2*-mediated maintenance of rDNA contributes to germline immortality.
174
175 Together, our findings reveal an unanticipated 'function' of retrotransposon activity to
176 benefit the host genome through a role in rDNA CN maintenance. Repetitive DNA sequences are
177 among the most vulnerable elements of the eukaryotic genome(*21*). We propose that DSBs
178 generated by *R2* in GSCs with reduced rDNA CN stimulate sister chromatid exchange that results
179 in rDNA CN expansion **(Fig. 4C)**. Our proposed 'function' for *R2* in rDNA maintenance may
180 represent a novel mutualistic host-TE relationship, which are rarely described in eukaryotes(*22*).
181 *R2*'s de-repression when it can be beneficial (i.e. decreased rDNA CN) may be the key to this
182 mutualistic host-TE relationship. It awaits future investigation to understand how the mechanisms
183 that repress *R2*(*10*) may tune this activity for host's benefit. The widespread presence of *R2* and
184 other rDNA-specific TEs in both vertebrates and invertebrates(*11*) suggests that similar host-TE
185 mutualism may support rDNA CN maintenance throughout Metazoa. Interestingly, many of rDNA-
186 specific TEs have little sequence similarity to *R2*, instead appearing to be derived from other non-
187 specific TEs(*11*), suggesting this host-TE mutualism may have evolved multiple times over the
188 course of evolution. In summary, our study provides an example of mutualistic retrotransposons
189 in the maintenance of eukaryotic genomes, and we propose that more mutualistic TEs are yet to
190 be discovered.
191
192 **Acknowledgements**

We thank the Bloomington *Drosophila* Stock Center, Kyoto *Drosophila* Stock Center and Developmental Studies Hybridoma Bank for reagents. We thank the Yamashita lab members and Dr. Andy Clark for discussion and comments on the manuscript. We thank ATCC for design of the 28S and RpL ddPCR assays. **Funding:** This research was supported by the Howard Hughes Medical Institute. Jonathan Nelson was supported by an American Cancer Society Postdoctoral Fellowship (133949-PF-19-133-01-DMC). **Authors contributions:** JON: Conceptualization, Methodology, Verification, Formal analysis, Investigation, Resources, Data curation, Writing – Original draft, Writing – Review & editing, Visualization, Funding acquisition. AS: Validation, Investigation, Resources, Data curation, Writing – Review & editing. YMY Conceptualization, Methodology, Resources, Writing – Original draft, Writing – Review & editing, Visualization, Supervision, Project administration, Funding. **Competing interests:** The authors declare no competing interests. **Data materials availability:** All data is available in the manuscript or the supplementary materials.

207 **References:**

208

209 1. A. F. Palazzo, E. S. Lee, Non-coding RNA: what is functional and what is junk? *Frontiers Genetics*. **6**,
210 2 (2015).

211 2. T. Kobayashi, Ribosomal RNA gene repeats, their stability and cellular senescence. *Proc Jpn Acad Ser*
212 *B*. **90**, 119–129 (2014).

213 3. B. L. Strehler, M.-P. Chang, L. K. Johnson, Loss of hybridizable ribosomal DNA from human post-
214 mitotic tissues during aging: I. Age-dependent loss in human myocardium. *Mech Ageing Dev*. **11**, 371–
215 378 (1979).

216 4. R. Johnson, B. L. Strehler, Loss of Genes coding for Ribosomal RNA in Ageing Brain Cells. *Nature*.
217 **240**, 412–414 (1972).

218 5. K. L. Lu, J. O. Nelson, G. J. Watase, N. Warsinger-Pepe, Y. M. Yamashita, Transgenerational
219 dynamics of rDNA copy number in Drosophila male germline stem cells. *Elife*. **7**, e32421 (2018).

220 6. J. O. Nelson, G. J. Watase, N. Warsinger-Pepe, Y. M. Yamashita, Mechanisms of rDNA Copy Number
221 Maintenance. *Trends Genet*. **35**, 734–742 (2019).

222 7. F. M. Ritossa, Unstable redundancy of genes for ribosomal RNA. *Proc National Acad Sci*. **60**, 509–516
223 (1968).

224 8. K. D. Tartof, Regulation of ribosomal RNA gene multiplicity in Drosophila melanogaster. *Genetics*.
225 **73**, 57–71 (1973).

226 9. R. S. Hawley, C. H. Marcus, Recombinational Controls of rDNA Redundancy in Drosophila. *Annu Rev*
227 *Genet*. **23**, 87–120 (1989).

228 10. T. H. Eickbush, D. G. Eickbush, Integration, regulation, and long-term stability of R2
229 retrotransposons. *Microbiol Spectr*. **3**, 1127–1146 (2015).

230 11. K. K. Kojima, H. Fujiwara, Long-Term Inheritance of the 28S rDNA-Specific Retrotransposon R2.
231 *Mol Biol Evol*. **22**, 2157–2165 (2005).

232 12. J. Yang, H. S. Malik, T. H. Eickbush, Identification of the endonuclease domain encoded by R2 and
233 other site-specific, non-long terminal repeat retrotransposable elements. *Proc National Acad Sci*. **96**,
234 7847–7852 (1999).

235 13. D. G. Eickbush, T. H. Eickbush, R2 Retrotransposons Encode a Self-Cleaving Ribozyme for
236 Processing from an rRNA Cotranscript ▿ . *Mol Cell Biol*. **30**, 3142–3150 (2010).

237 14. M. T. Fuller, A. C. Spradling, Male and Female Drosophila Germline Stem Cells: Two Versions of
238 Immortality. *Science*. **316**, 402–404 (2007).

239 15. D. L. Lindsley, L. Sandler, The Meiotic Behavior of Grossly Deleted X Chromosomes in Drosophila
240 Melanogaster. *Genetics*. **43**, 547–63 (1958).

241 16. F. M. Ritossa, K. C. Atwood, S. Spiegelman, A molecular explanation of the bobbed mutants of
242 Drosophila as partial deficiencies of "ribosomal" DNA. *Genetics*. **54**, 819–34 (1966).

243 17. S. Gangloff, H. Zou, R. Rothstein, Gene conversion plays the major role in controlling the stability of
244 large tandem repeats in yeast. *Embo J*. **15**, 1715–1725 (1996).

245 18. K. D. Tartof, Unequal Mitotic Sister Chromatid Exchange as the Mechanism of Ribosomal RNA
246 Gene Magnification. *Proc National Acad Sci*. **71**, 1272–1276 (1974).

247 19. S. Paredes, K. A. Maggert, Expression of I-CreI Endonuclease Generates Deletions Within the rDNA
248 of Drosophila. *Genetics*. **181**, 1661–1671 (2009).

249 20. C. Smelick, S. Ahmed, Achieving immortality in the C. elegans germline. *Ageing Res Rev*. **4**, 67–82
250 (2005).

251 21. R. E. Brown, C. H. Freudenreich, Structure-forming repeats and their impact on genome stability.
252 *Curr Opin Genet Dev*. **67**, 41–51 (2021).

253 22. R. L. Cosby, N.-C. Chang, C. Feschotte, Host–transposon interactions: conflict, cooperation, and
254 cooption. *Gene Dev*. **33**, 1098–1116 (2019).

255
256

List of Supplementary Materials

Supplementary Materials

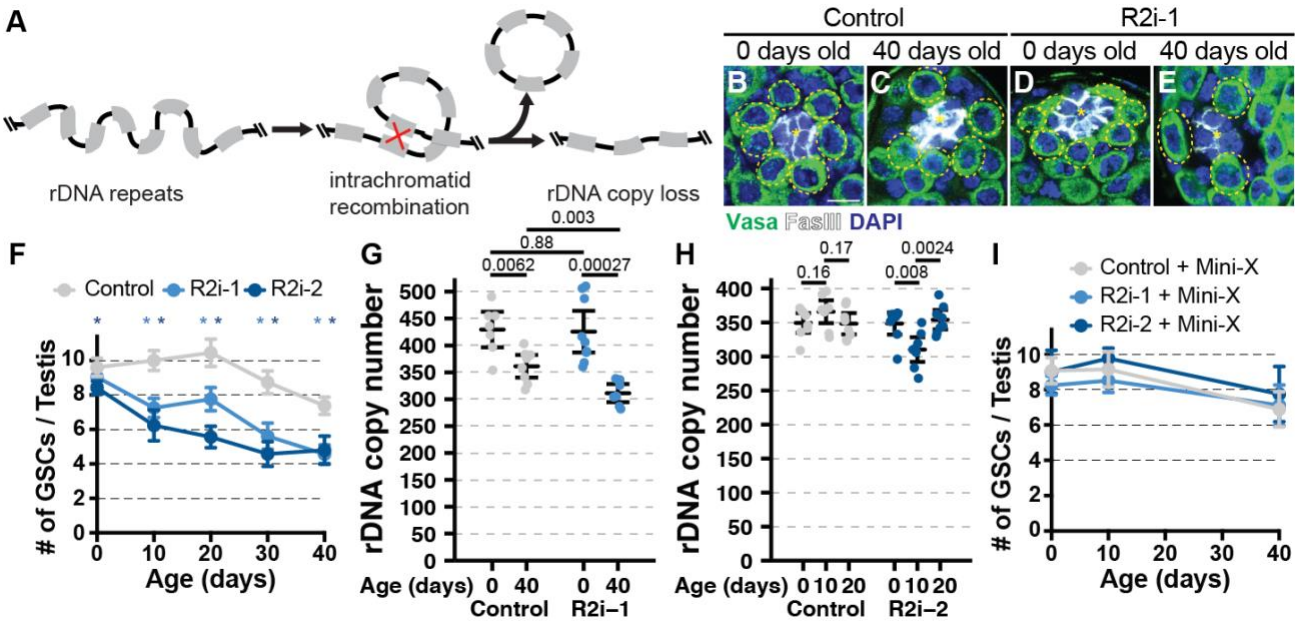
Materials and Methods

Fig S1 – S8

Table S1 – S2

References 23 - 30

264



265

266 **Figure 1: *R2* is required for GSC maintenance via rDNA CN maintenance during**

267 ***Drosophila* male germline aging. (A)** Model of rDNA repeat instability. **(B-E)** GSCs in 0- and

268 40-day old control (B-C) and *R2* RNAi testes (D-E). Yellow dotted circle = GSC. GSC signaling

269 niche indicated by *. Green = Vasa, White = Fascillin III, Blue = DAPI. Scale bar = 7.5 μM **(F)**

270 Average GSCs per testis in control and two *R2* RNAi lines during aging. * indicates $p < 10^{-3}$

271 determined by Student's t-test. Error = 95% confidence interval (CI). **(G-H)** Testis rDNA CN

272 determined by ddPCR. P-value by Student's t-test. Error = 95% CI. **(I)** Average GSCs per testis

273 during aging in Control and two *R2* RNAi lines containing mini-X chromosome. Error = 95% CI.

274

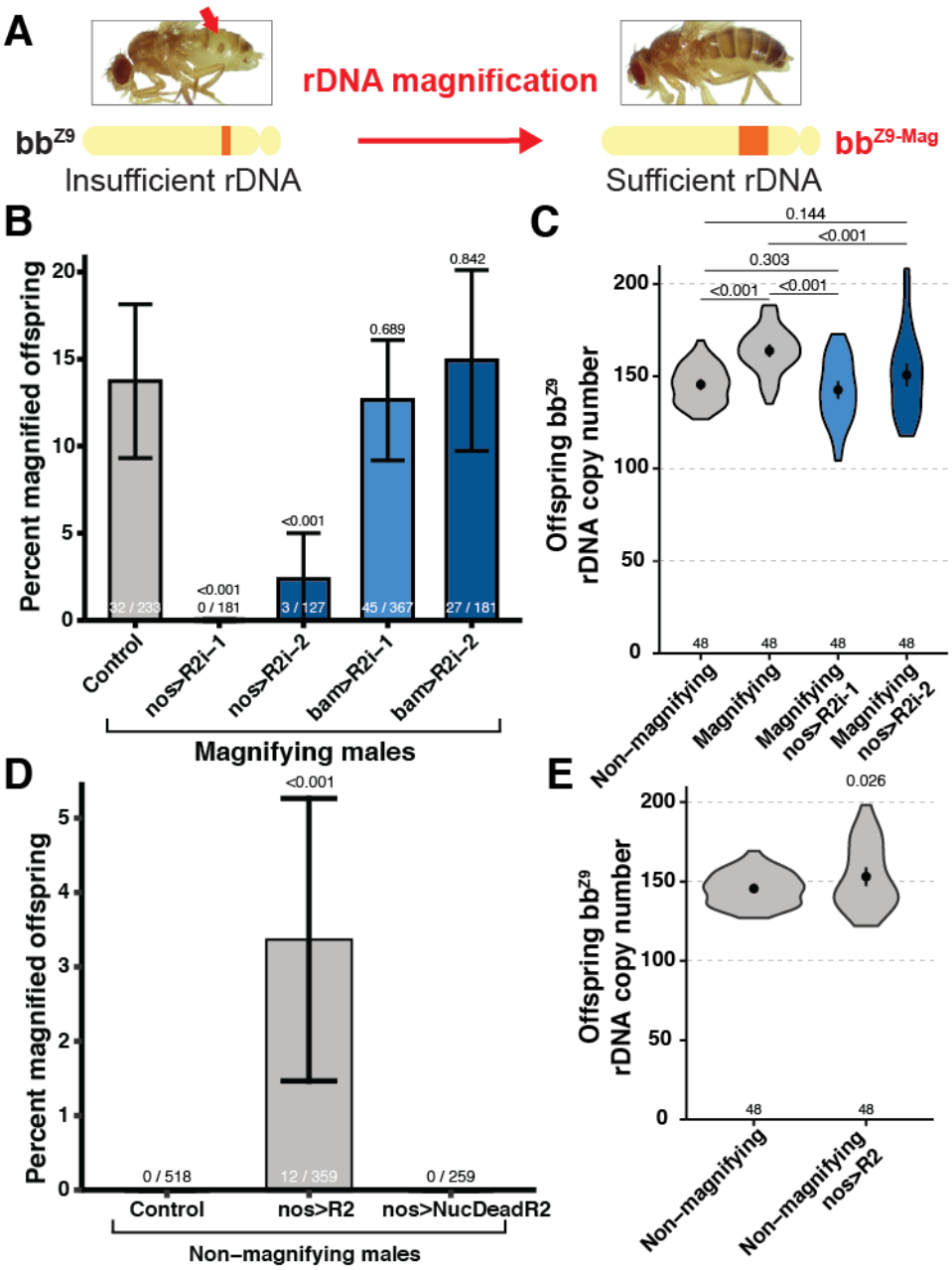


Figure 2: *R2* is necessary and sufficient for rDNA magnification. (A) Diagram of rDNA magnification at the *bb*^{Z9} rDNA locus, during which dorsal cuticle defect (red arrow) revert to normal cuticle. **(B)** Percent magnified offspring determined by cuticular phenotype in offspring from magnifying males. P-value determined by chi-squared test. Error = 95% CI. **(C)** Mean *bb*^{Z9} locus rDNA CN determined by ddPCR in daughters from males. P-value determined by Student's t-test. Error = 95% CI. **(D)** Percent magnified offspring from non-magnifying males. P-

282 value determined by chi-squared test. Error = 95% CI. **(E)** Mean bb^{Z9} locus rDNA CN

283 determined by ddPCR in daughters from non-magnifying males. Non-magnifying condition is the

284 same data as panel (C). P-value determined by Student's t-test. Error = 95% CI.

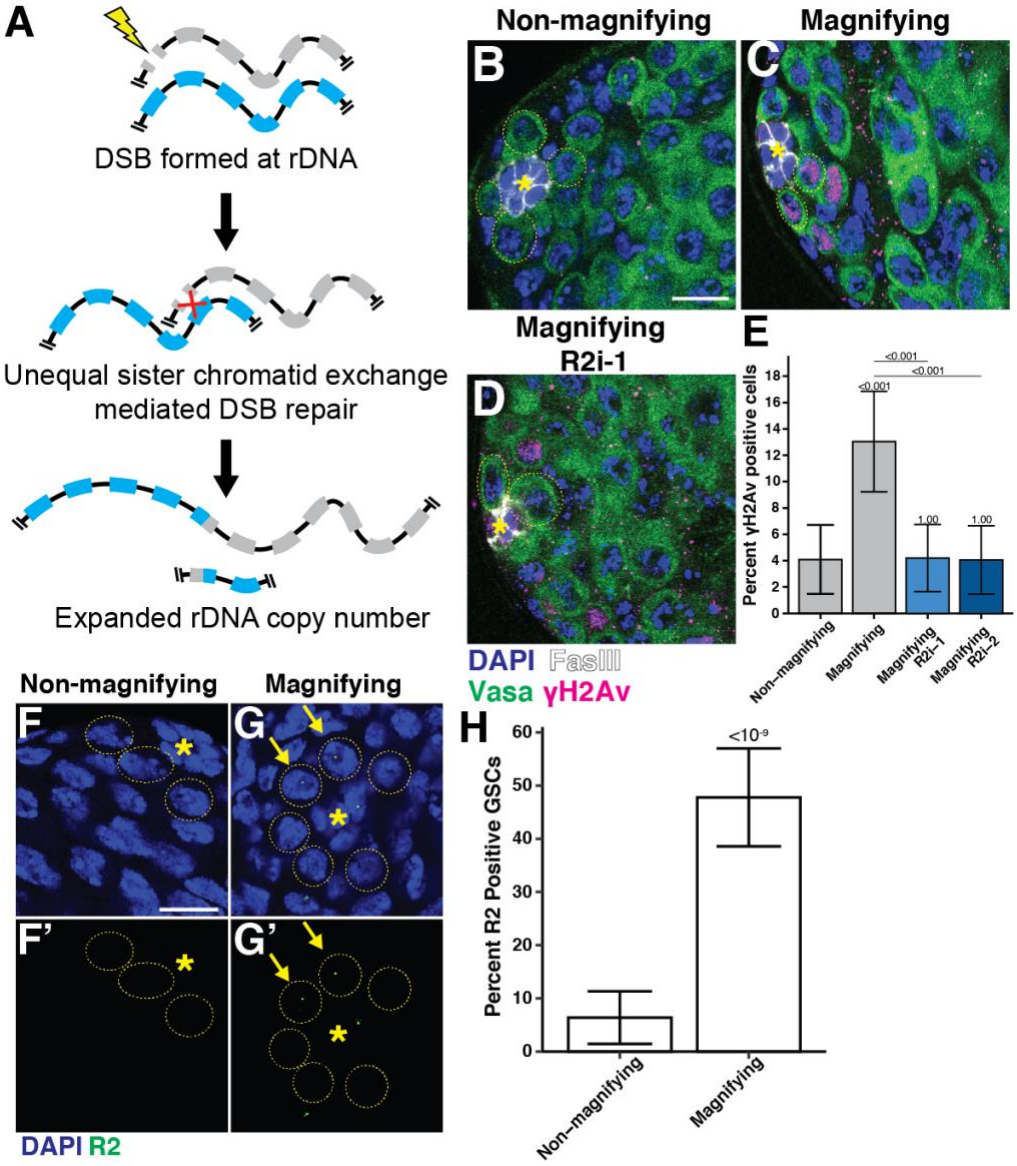


Fig 3: Derepressed *R2* creates DSBs in GSCs during rDNA magnification. (A) Diagram of rDNA CN expansion by unequal sister chromatid exchange during DSB repair at rDNA loci. Recombination between misaligned rDNA copies during DSB repair result in crossovers that create unequal sister chromatid exchange that increases rDNA CN on one chromatid. **(B-D)** Detection of DSBs in the early adult male germline by anti-γH2Av staining. *R2* RNAi expressed under the *nos-gal4* driver. Non-RNAi conditions contain the *nos-gal4* driver alone. GSCs indicated by yellow dotted circle. Blue = DAPI, Green = vasa, Magenta = γH2Av, white = FasIII. The hub is indicated by *. Scale bar = 10 μM. **(E)** Percentage of γH2Av positive GSCs. P-value

294 determined by chi-squared test. Error = 95% CI. **(F-G)** *R2* expression in GSCs (yellow dotted

295 circle). Blue = DAPI, Green = *R2* mRNA. Isolated *R2* channel in F'-G'. The hub is indicated by *.

296 *R2* positive cells GSCs are marked by yellow arrowhead. **(H)** Percentage of *R2* positive GSCs.

297 P-value determined by chi-squared test. Error = 95% CI.

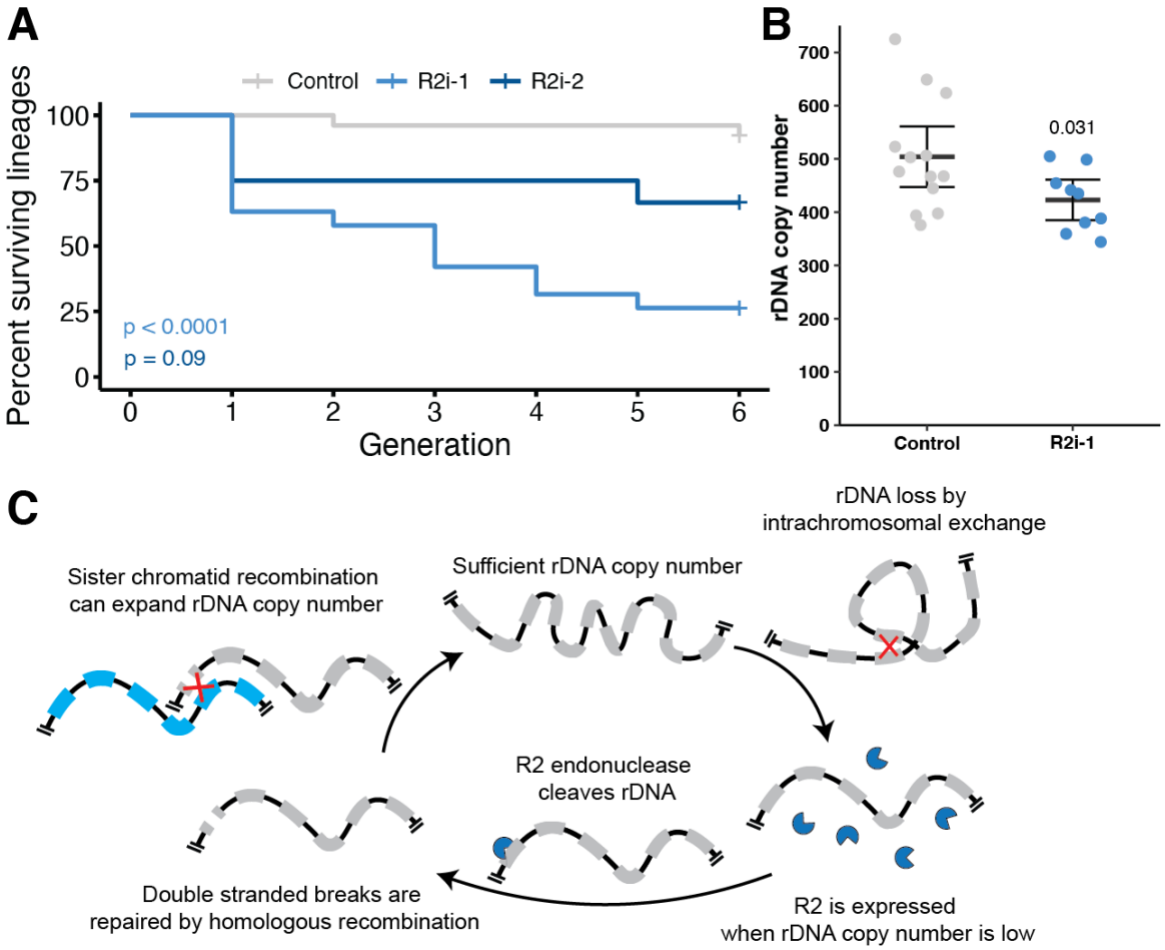


Fig 4: *R2* is required to maintain rDNA CN and fertility over successive generations. (A) Kaplan-Meier curve of lineage survival in control (*nos-gal4* driver alone and two *R2* RNAi expressing (via the *nos-gal4* driver) lineages. Each lineage constitutes an individual data point. P-values determined by log rank test. **(B)** rDNA CN determined by ddPCR in males of control animals at the 6th generation or *R2* RNAi lineages at their terminating generation. P-value determined by Student's t-test. Error = 95% CI. **(C)** Model of the role of *R2* in germline rDNA CN maintenance.