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.

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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 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 copy number (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 retrotransposons can provide a benefit to their hosts, contrary to their reputation as genomic parasitic, 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*, *6*). rDNA CN is variable between individuals of most species but maintained within a consistent range throughout the population(*7*), implying that trans-generational dynamic rDNA CN changes (loss and restoration) are a common feature of rDNA maintenance. However, the mechanism of trans-

generational rDNA CN maintenance in multicellular organisms has remained a mystery. Over 50 years ago, the phenomenon of 'rDNA magnification' in Drosophila was first described as the process wherein aberrant rDNA loci bearing minimal rDNA repeats recover to a normal rDNA CN(*8*). rDNA magnification was shown to require genes involved in homologous recombination-mediated repair, similar to yeast, which can create duplications of tandemly repeated elements(*9*). Despite the robust rDNA CN expansion activity, it remained unclear if the mechanisms of rDNA magnification served a physiological function in natural populations. We recently demonstrated that offspring who inherited reduced rDNA CN from old fathers could also recover normal rDNA CN in their germline(*5*). This recovery depends on the same set of the genes as rDNA magnification, leading us to propose that rDNA magnification is a manifestation of the physiological mechanisms to maintain rDNA CN across generations(*7*). However, the underlying factors responsible for rDNA magnification and their normal physiological functions remain poorly understood.

Metazoan rDNA genes are frequently inserted by rDNA-specific transposable elements (TEs), such as the retrotransposon *R2* in Drosophila. *R2* is found throughout arthropods(*10*) and *R2*-like elements are widely present across taxa, including Cnidaria(*11*), Planaria(*12*), nematodes(*13*), fish(*14*, *15*), birds, and reptiles(*15*). These TEs use their sequence-specific nuclease to mobilize specifically within rDNA loci, inserting in rDNA genes and disrupting their function(*16*) **(Fig. S1A)**. Surprisingly, we found that RNAi-mediated knockdown of *R2* in the Drosophila male germline (nos-gal4 driving *R2* RNAi expression) **(Fig. S1A-B)** resulted in premature loss of germline stem cells (GSCs) during aging **(Fig. 1B-F)**. GSCs continuously produce differentiating germ cells to sustain sperm production throughout adulthood and thus are the source of the genome passed to the next generation(*17*). Whereas newly eclosed *R2* RNAi males contained similar numbers of GSCs to controls, GSC number more rapidly declined during aging in *R2* knockdown males compared to controls **(Fig. 1B-F)**. Given that *R2* is specifically

inserted at rDNA, we also examined the effect of *R2* knockdown on rDNA stability. Using highly quantitative droplet digital PCR (ddPCR), we found that RNAi-mediated knockdown of *R2* enhanced rDNA CN loss in the testis during aging compared to controls **(Fig. 1G)**. rDNA CN insufficiency is likely the primary cause of GSC loss in *R2* RNAi animals, because increasing total rDNA CN via introduction of a mini-chromosome harboring an rDNA locus(*18*) suppressed the premature GSC loss caused by *R2* knockdown **(Fig. 1H)**. These results revealed that *R2* contributes to sustaining GSC population during aging by supporting rDNA CN maintenance, uncovering an unanticipated benefit of the R2 retrotransposon to the host, despite the widely-held view of TEs being genetic parasites.

Why is *R2* necessary for rDNA copy number maintenance? We found that *R2* is required for rDNA magnification, a phenomenon of rDNA CN expansion classically assayed by the restoration of normal cuticular development in animals inheriting rDNA loci that had insufficient rDNA CN in the previous generation **(Fig. 2A)**. The *Drosophila melanogaster* genome has two rDNA loci, one on each X and Y chromosome(*19*). rDNA magnification almost exclusively occurs to X chromosome rDNA loci harboring the minimal viable amount of rDNA (bb^{Z9}, **Fig. S2A)** when combined with a Y chromosome completely lacking rDNA (bb^{Z9}/Ybb^{0}, 'magnifying males' hereafter)(*8*) **(Fig. S2B)**. Importantly, rDNA magnification never occurs in males with a normal Y chromosome containing intact rDNA (bb^{Z9}/Y^{+}, 'non-magnifying males' hereafter)(*20*), indicating the presence of mechanisms to monitor rDNA CN to activate expansion. We found that RNAi-mediated knockdown of *R2* eliminated rDNA magnification observed in magnifying males from 13.73% to 0% ($n = 414$, $p = 5.6\times10^{-7}$) **(Fig. 2B)**. Moreover, quantification of rDNA CN by ddPCR revealed that 87.5% of bb^{Z9} chromosomes increased rDNA CN in magnifying males compared to the non-magnifying males ($n = 96$, $p = 1.8\times10^{-4}$), with an average increase of 18.29 rDNA copies across all samples ($n = 96$, $p = 3.1\times10^{-12}$) **(Fig. 2C)**. This widespread rDNA CN increase indicates that rDNA magnification broadly increases rDNA CN throughout the germline, despite only

13.73% of bb^{Z9} chromosomes having enough CN increase to support normal cuticle development. We found this rDNA CN increase is also eliminated upon *R2* knockdown in magnifying males (n = 96, p = 4.7×10^{-10}) **(Fig. 2C)**. These results reveal that *R2* is required for rDNA CN expansion during rDNA magnification. Interestingly, we found that *R2* knockdown only disrupted rDNA magnification when the *R2* RNAi construct was expressed by the *nanos*-gal4 driver in early germ cells (including GSCs)(*21*), but not when expressed in later germ cells (excluding GSCs) by the bam-gal4 driver(*22*) **(Fig. 2B)**. These results indicate that *R2* primarily functions in the earliest stages of germ cells (including GSCs) to support rDNA magnification.

We further found that *R2* is sufficient for rDNA CN expansion. Ectopic expression of *R2* in the germline induced rDNA magnification of the bb^{Z9} locus in non-magnifying males (bb^{Z9}/Y^{+}) **(Fig. 2D-E)**. While assessment of dorsal cuticle phenotype never detects rDNA magnification from control males, we found 3.3% of bb^{Z9} rDNA loci from non-magnifying males expressing a transgenic copy of *R2* were phenotypically magnified **(Fig. 2D,** n = 877, p = 3.2×10^{-5}). Quantification of rDNA CN by ddPCR revealed that ectopic overexpression of *R2* in non-magnifying males (bb^{Z9}/Y^{+}) also increases the average rDNA CN at bb^{Z9} rDNA loci among all offspring compared to controls **(Fig. 2E**, n = 94, p = 0.0256), revealing *R2* is sufficient to induce rDNA CN expansion. Critically, expression of a nuclease dead R2 transgene (NucDeadR2) in non-magnifying males **(Fig. S3A)**(*23*) failed to induce phenotypic detection of rDNA magnification **(Fig. 2D)**, suggesting that the nuclease activity of R2 is essential for its ability to induce rDNA CN expansion.

How does R2's nuclease activity contribute to rDNA magnification? In yeast, rDNA CN expansion is initiated by double-stranded breaks (DSBs) at the rDNA intergenic sequence, which induces sister chromatid recombination that results in rDNA gene duplication(*24*). While the mechanism of rDNA magnification remains unclear, all proposed models of Drosophila rDNA CN

expansion require an initiating DSB at the rDNA locus, the most prominent model being sister chromatid recombination resulting in unequal sister chromatid recombination(*25*) **(Fig. 3A; Fig. S4A-B** for additional models**)**. Indeed, artificial introduction of DSBs at rDNA loci by I-CreI endonuclease expression has been reported to induce rDNA magnification(*26*), but the endogenous factor that induces rDNA magnification remained unclear. R2 is capable of creating DSBs through sequential nicking of opposite DNA strands during retrotransposition(*27*). It has been speculated that DSBs created during R2 retrotranspostion may be an initiating event of rDNA magnification(*28*), although this possibility has yet to be empirically tested. We first confirmed that ectopic overexpression of R2, but not NucDeadR2, indeed induces chromosomal breaks at rDNA loci identified by chromosome spreads **(Fig. S3B-D)**. R2 overexpression (but not NucDeadR2) in the germline also increased the frequency of GSCs with DSBs, identified by γH2Av expression **(Fig. S3E)**. Next, we found rDNA magnification is associated with an elevation in DSBs in GSCs: the frequency of γH2Av-positive GSCs is increased in magnifying males (bb^{Z9}/Ybb^{0}) compared to non-magnifying males (bb^{Z9}/Y^{+}) (**Fig. 3B-C, E**; n = 519, p = 8.8×10^{-4}). Strikingly, we observed that knockdown of *R2* in magnifying males reduced the frequency of γH2Av-positive GSCs to levels comparable to non-magnifying males **(Fig. 3D-E;** n = 537, p = 7.1×10^{-4}**)**, indicating that R2 is responsible for the DSBs formed in GSCs during rDNA magnification. Taken together, these results suggest that rDNA-specific endonuclease activity of R2, which functions during R2 retrotransposition, creates DSBs at the rDNA loci that may in turn induce rDNA CN expansion.

Given the threat *R2* mobilization poses to the host genome, both by disruption of rRNA function and causing excessive DSB formation(*27*), how is the potential benefit of *R2* to rDNA CN maintenance balanced with the detriment of *R2* retrotransposition? We found *R2* expression in the germline is specifically de-repressed under conditions of reduced rDNA CN, potentially

explaining how the conflicting consequences of *R2* expression are resolved. Using RNA fluorescence *in situ* hybridization (RNA FISH) to examine *R2* expression at a single cell resolution, we found that the frequency of GSCs expressing *R2* was significantly increased in magnifying males (bb^{Z9}/Ybb^{0}), whereas non-magnifying males (bb^{Z9}/Y^{+}) rarely expressed *R2* **(Fig. 3F-H**; n = 231, p = $1.7x10^{-10}$). Moreover, we found that GSCs from aged animals and the sons of old fathers, which inherit reduced rDNA CN(*5*)**,** also exhibited a higher frequency of *R2* expression compared to GSCs from young, wild type flies **(Fig. S5A-B, D**; n = 1,247, p = $8.3x10^{-4}$ for old animals; n = 1,107, p = $1.5x10^{-4}$ for offspring). Importantly, the frequency of *R2* expression among GSCs in the sons of old fathers returned to the basal level after 20 days of age, when rDNA CN was shown to have recovered(*5*) **(Fig. S5C-D**; n = 617, p = 0.036**)**. These results indicate that *R2* expression is dynamically regulated in response to changing rDNA CN. Taken together, we propose that *R2* expression is finely tuned to function when most beneficial to the host while minimizing unnecessary exposure to the harmful effects of transposition.

Based on the finding that *R2* plays a critical role in maintaining germline rDNA CN, we postulated that *R2* is essential to prevent continuous multi-generational rDNA loss capable of causing the extinction of the lineage. In *C. elegans*, the loss of genome integrity is known to cause gradual loss of fertility, a phenotype known as mortal germline (morg)(*29*). To test whether *R2*-mediated rDNA maintenance is required to maintain fertility through generations, we established multiple independent lines expressing *R2* RNAi in their germline and tracked their fecundity at each generation through the ability of each line to produce sufficient offspring to establish a new generation **(Fig. S6)**. We found many *R2* RNAi lines failed to produce sufficient progeny, with over half failing by the fourth generation, while nearly all control lines survived throughout the duration of the experiments **(Fig. 4A)**, indicating that *R2* is essential for continuity of the germline lineage. Males of extinguishing *R2* RNAi lineages had ~20% reduction in rDNA CN compared to

control lines (n = 22, p = 0.031) **(Fig. 4B)**. These results suggest that *R2*-mediated maintenance of rDNA contributes to germline immortality.

Together, our findings reveal an unanticipated 'function' of retrotransposons to benefit the host genome through a role in rDNA CN maintenance. Repetitive DNA sequences such as rDNA are among the most vulnerable elements of the eukaryotic genome(*30*, *31*). We propose that DSBs generated by *R2* in GSCs with diminished rDNA loci stimulate sister chromatid recombination that results in rDNA CN expansion **(Fig. 4C)**. Our proposed 'function' for *R2* in rDNA maintenance may represent a novel mutualistic host-TE relationship, which are rarely described in eukaryotes(*32*). *R2*'s de-repression when it can be beneficial (i.e. decreased rDNA CN) may be the key to this mutualistic host-TE relationship. It awaits future investigation to understand how the mechanisms that repress *R2*(*33*–*35*) may tune this activity for host's benefit. The widespread presence of *R2* and other rDNA-specific TEs in both vertebrates and invertebrates(*15*) suggests that similar host-TE mutualism may support rDNA CN maintenance throughout Metazoa. Interestingly, many of these other *R2*-like elements have little sequence similarity to R2, instead appearing to be derived from other non-specific TEs(*15*), suggesting this host-TE mutualism may have evolved multiple times over the course of evolution. In summary, our study provides an example of beneficial TEs in the maintenance of eukaryotic genomes, and we propose that more beneficial TEs are yet to be discovered.

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List of Supplementary Materials

Supplementary Materials

Materials and Methods

Fig S1 – S6

Table S1 – S2

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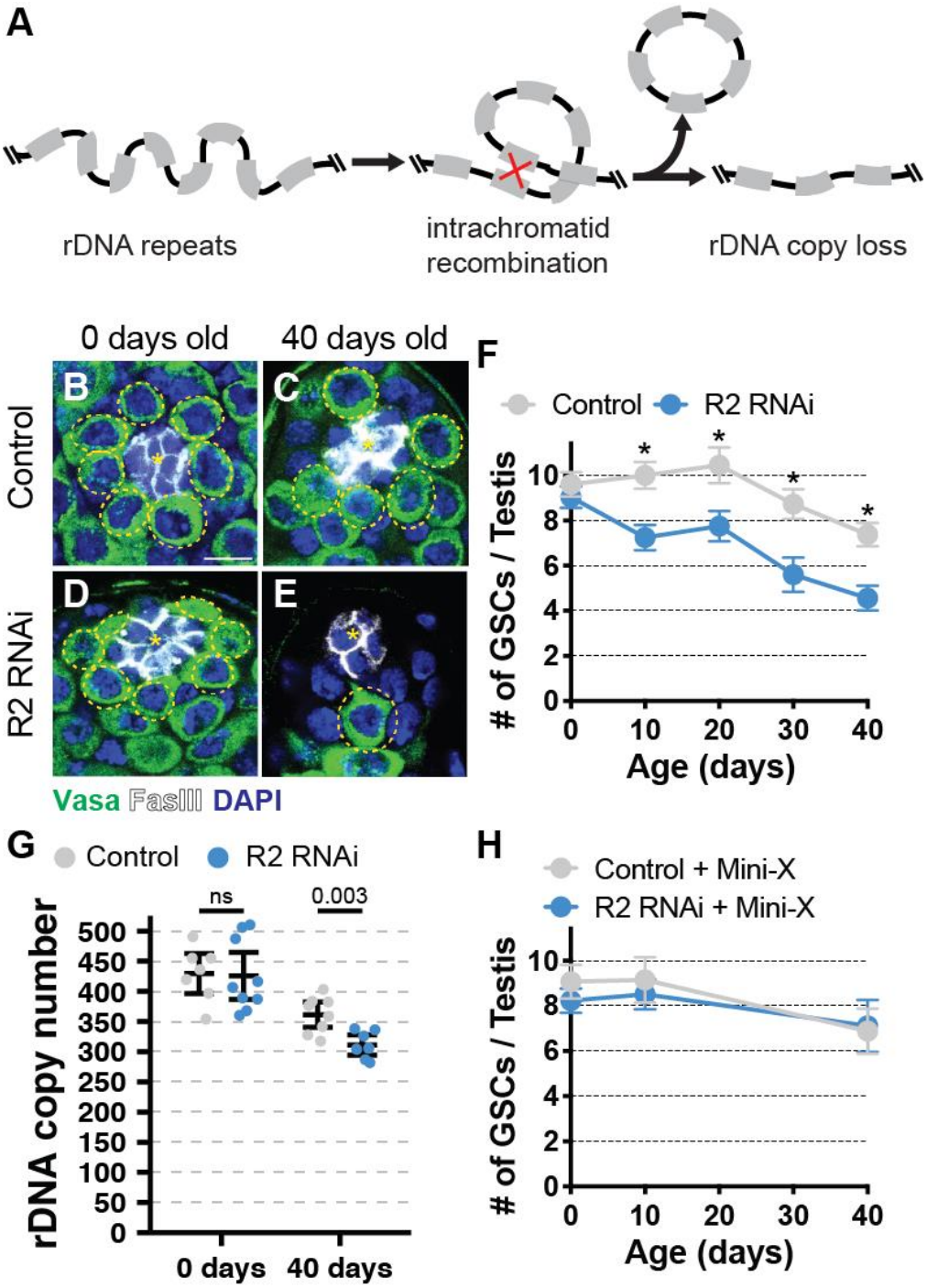

Figure 1: R2 is required for GSC maintenance via rDNA CN maintenance during Drosophila male germline aging. (A) Tandem rDNA copy repeats are lost through circularization and extrusion caused by intra-chromatid recombination between rDNA copies.

(B-E) GSCs in 0- and 40-day old control (*nos-gal4* driver alone) (B-C) and R2 RNAi (*nos-gal4* driving *UAS-R2 RNAi*) testes (D-E). Yellow dotted circle = GSC. The major component of the GSC signaling niche, the hub, is indicated by *. Green = Vasa, White = Fascillin III, Blue = DAPI. Scale bar = 7.5 µM **(F)** Average number of GSCs per testis in control and R2 RNAi animals during aging. * indicates $p < 10^{-5}$ determined by Student's t-test. Error bars = 95% confidence interval (CI). **(G)** rDNA CN determined by ddPCR in testes of 0- and 40-day old control and R2 RNAi animals. P-value determined by Student's t-test. Error bars = 95% CI. **(H)** Average number of GSCs per testis during aging in Control and R2 RNAi animals with mini-X chromosome. Error bars = 95% CI.

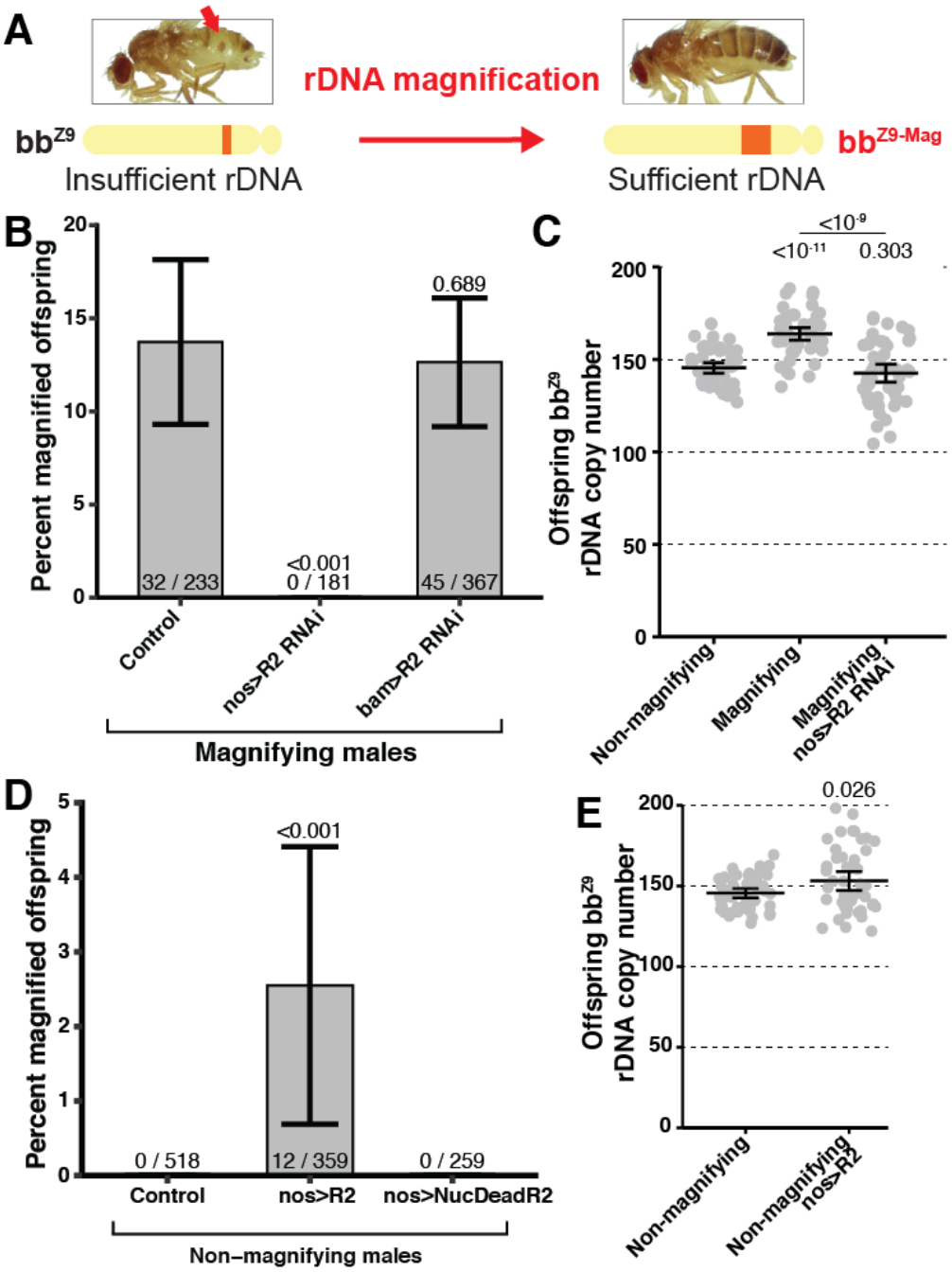

Figure 2: R2 is necessary and sufficient for rDNA magnification. (A) Diagram of rDNA magnification. Animals with insufficient rDNA CN due to having the bb^{Z9} locus as their only source of rDNA have distinct developmental defects resulting in gaps in the dorsal cuticle (red arrow). rDNA CN expansion at the bb^{Z9} locus due to rDNA magnification is detected by reversion of this phenotype to normal cuticles. **(B)** Percent magnified offspring determined by

cuticular phenotype in offspring from magnifying males of the indicated genotypes. P-value determined by chi-squared test. Error bars = 95% CI. **(C)** bb^{Z9} locus rDNA CN determined by ddPCR in daughters from males of indicated genotypes. P-value determined by Student's t-test. Error bars = 95% CI. Non-magnifiying condition is the same data as panel (C). **(D)** Percent magnified offspring from non-magnifying males of the indicated genotypes. P-value determined by chi-squared test. Error bars = 95% CI. **(E)** bb^{Z9} locus rDNA CN determined by ddPCR in daughters from non-magnifying males of the indicated genotype. P-value determined by Student's t-test. Error bars = 95% CI.

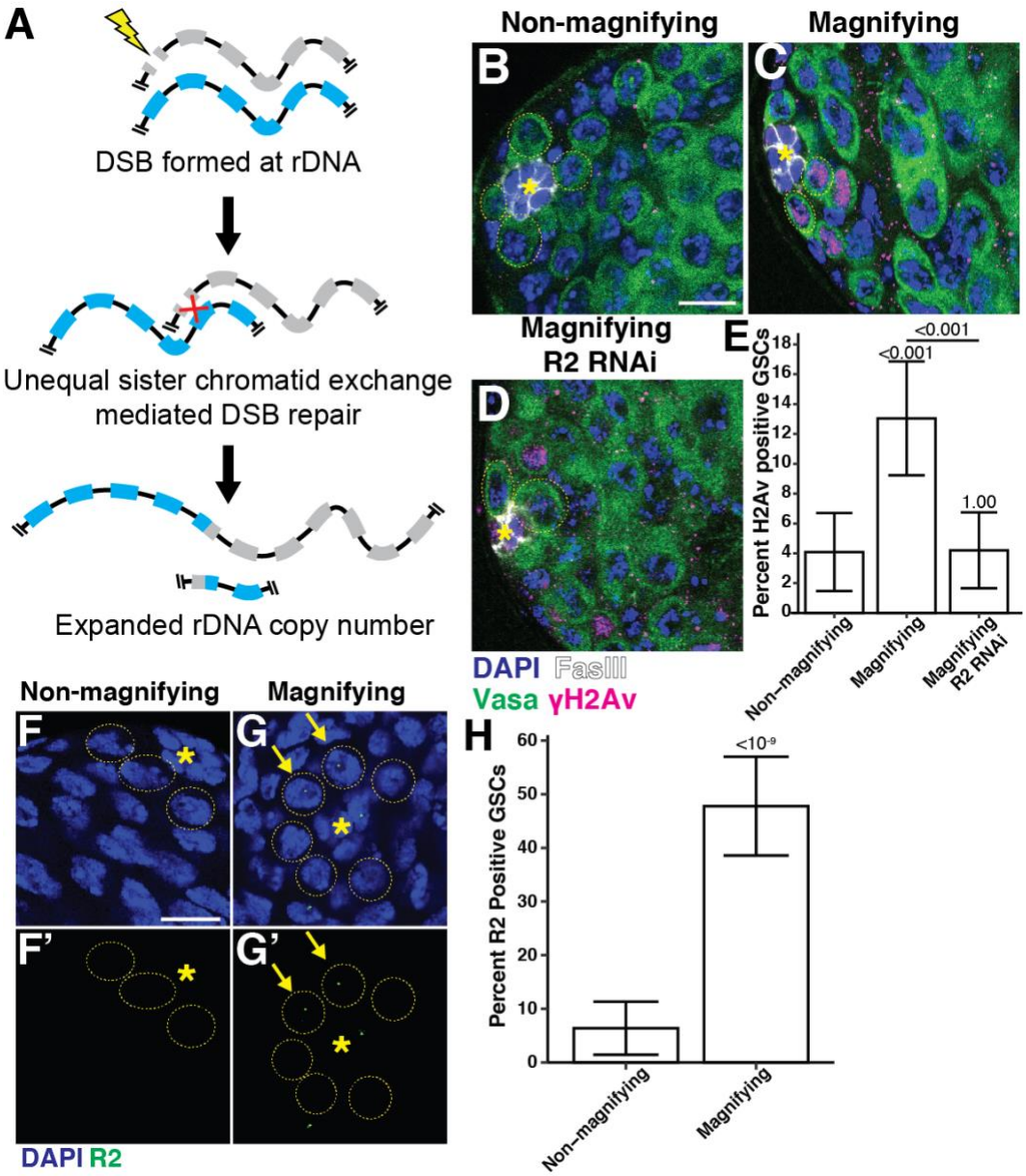

Fig 3: Derepressed R2 creates DSBs in GSCs during rDNA magnification. (A) Diagram of rDNA CN expansion by unequal sister chromatid exchange during DSB repair at rDNA loci. Recombination between misaligned rDNA copies during DSB repair result in crossovers that create unequal sister chromatid exchange 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 in the indicated conditions. P-value determined by chi-squared test. Error bars = 95% CI. **(F-G)** R2 expression in GSCs (yellow dotted circle). Blue = DAPI, Green = R2 mRNA. Isolated R2 channel in F'-G'. The hub is indicated by *. R2 positive cells GSCs are marked by yellow arrowhead. **(H)** Percentage of R2 positive GSCs. P-value determined by chi-squared test. Error bars = 95% CI.

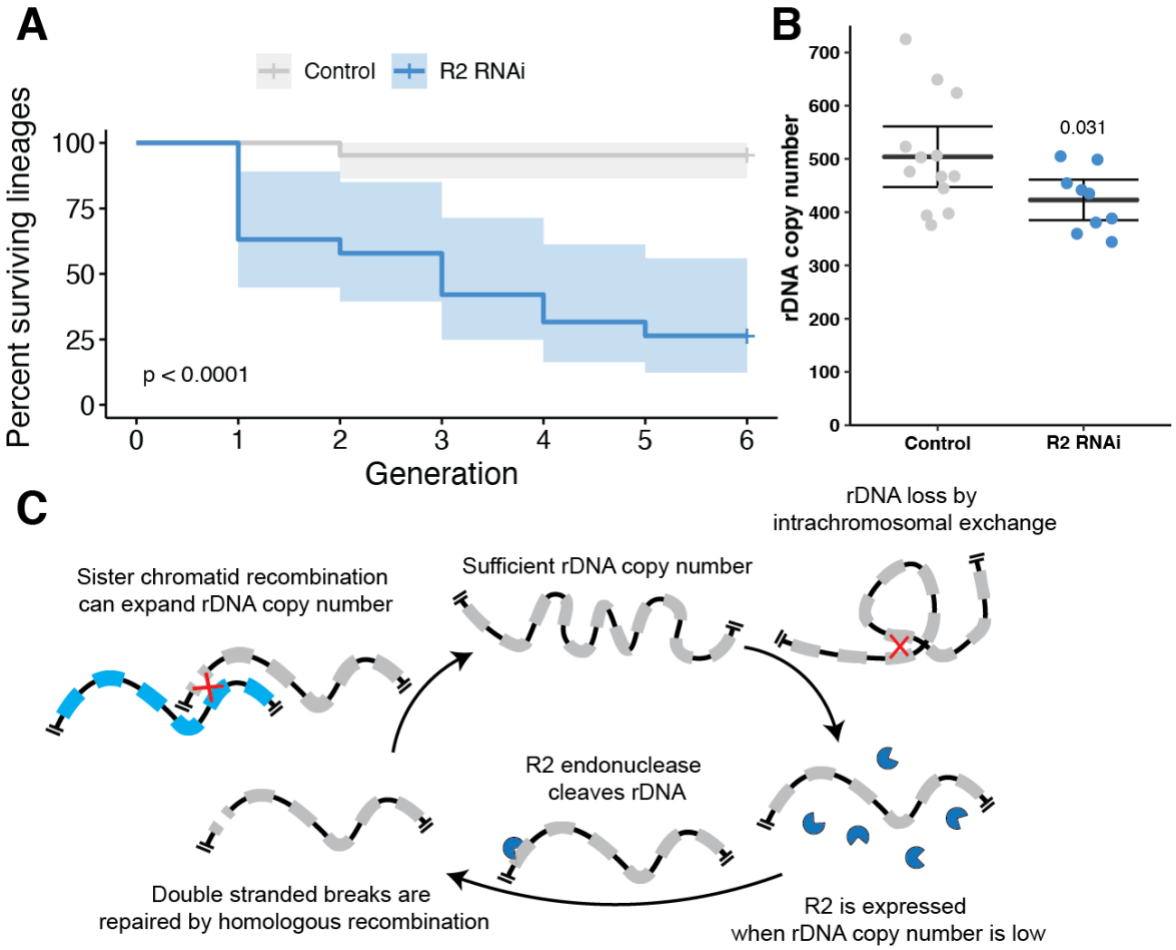

Fig 4: R2 is required to maintain rDNA CN and fertility over successive generations. (A) Kaplan-Meier curve of lineage survival in R2 RNAi and control lineages. Each lineage constitutes an individual data point. P-value determined by log rank test. Shade represents 95% CI. **(B)** rDNA CN determined by ddPCR in males of control (*nos-gal4* driver alone) animals at the 6th generation or R2 RNAi (RNAi expressed under the *nos-gal4* driver) 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. R2 derepression in cells with reduced rDNA CN leads to DSBs formed by rDNA-specific R2 endonuclease that can induce rDNA CN expansion through sister chromatid recombination during their repair.

Supplementary Materials

Materials and Methods

Immunofluorescence

Immunofluorescence staining of testes was performed as previously described(*36*). Briefly, testes were dissected in PBS, fixed in 4% formaldehyde in PBS for 30 minutes, then briefly washed two times in PBS containing 0.1% Triton-X (PBS-T), followed by washing in PBS-T for 30 minutes. After washes, samples were incubated at 4°C overnight with primary antibody in 3% bovine serum albumin (BSA) in PBS-T. Samples were washed three consecutive times for 20 minutes in PBS-T, then incubated at 4°C overnight with secondary antibody in 3% BSA in PBS-T, washed three times again in PBS-T for 20 minutes, and mounted in VECTASHIELD with DAPI (Vector Labs). The following primary antibodies were used: rat anti-vasa (1:20; DSHB; developed by A. Spradling), mouse anti-Fascillin III (1:200; DSHB; developed by C. Goodman), and rabbit anti-γ-H2AvD pS137 (1:200; Rockland). Images were taken with a Leica Stellaris 8 confocal microscope with 63x oil-immersion objectives and processed using Fiji (ImageJ) software.

RNA FISH

RNA FISH samples were prepared as previously described(*5*). In short, dissected testes were fixed in 4% formaldehyde in PBS for 30 minutes, briefly washed in PBS, and permeabilized in 70% ethanol overnight at 4º. Samples were then briefly rinsed in 2xSSC with 10% formamide prior to hybridization with 50nM Stellaris probes overnight at 37º. Samples were washed twice in 2xSSC with 10% formamide for 30 minutes and mounted in VECTASHIELD with DAPI (Vector Labs). Samples were imaged using a Leica Stellaris 8 confocal microscope with 63x oil-immersion objectives and processed using Fiji (ImageJ) software.

DNA isolation

Testis DNA was isolated from 50 pooled dissected testes frozen in liquid N2. DNA isolation was performed according to previously described methods for isolation from Drosophila tissues(*37*). DNA was isolated from individual Drosophila animals using a modified protocol of the DNeasy Blood and Tissue DNA extraction kit (Qiagen). In short, individual animals were homogenized in 200 µL Buffer ATL containing proteinase K using a pipette tip in Eppendorf tubes, vortexed for 15 seconds, and incubated for 1.5 hours at 56º. Samples were then prepared following the manufacturer's protocol after incubation. All DNA samples were quantified and checked for purity by Nanodrop One spectrophotometer (ThermoFisher).

rDNA copy number measurement by Droplet Digital PCR (ddPCR)

30 ng of DNA sample were used per 20 µL ddPCR reaction for control gene reactions (RpL and Upf1), and 0.3 ng of DNA per 20 µL ddPCR reaction for 28S rDNA reactions. Primers and probes for reactions are listed at **Table S1**. ddPCR reactions were carried out according to the manufacturer's (Bio Rad) protocol. In short, master mixes containing ddPCR Supermix for Probes (No dUTP) (Bio Rad), DNA samples, primer / probe mixes, and HindIII-HF restriction enzyme (New England Biolabs) for 28S rDNA reactions (no restriction enzyme for control gene reactions) were prepared in 0.2 mL Eppendorf tubes, and incubated at room temperature for 15 minutes to allow for restriction enzyme digestion. ddPCR droplets were generated from samples using QX200 Droplet Generator (Bio Rad) and underwent complete PCR cycling on a C100 deep-well thermocycler (Bio Rad). Droplet fluorescence was read using the QX200 Droplet Reader (Bio Rad). Sample copy number was determined using Quantasoft software (Bio Rad). rDNA copy number per genome was determined by 28S sample copy number multiplied by 100 (due to the 100x dilution of sample in the 28S reaction compared to control reaction) divided by control gene copy number multiplied by the expected number of control gene copies per genome (2 for RpL in all samples; 2 for Upf1 in female samples; 1 for Upf1 in male samples). The 28S copy number

values determined by each control gene was averaged to determine 28S copy number for each sample.

Mitotic chromosome spread and DNA FISH

Larval brain dissections, mitotic chromosome spreads, DNA FISH, and imaging were all done as previously(*38*). In short, brains were dissected from male third instar larvae in PBS and fixed in 25µ µL of acetid acid and 4% formaldehyde in PBS. Samples were applied to Superfrost plus slides and manually squashed under a coverslip, then immediately frozen in liquid N2. After freezing slides were removed from N2, coverslip removed, and slides were dehydrated in 100% ethanol and dried at room temperature. DNA FISH hybridization was performed in 20 µL of 50% formamide, 10% dextran sulfate, 2X SSC buffer and 0.5 µM each probe applied directly to the sample on the slide and covered with a cover slip. Samples were incubated at 95° for 5 minutes, cooled and wrapped in parafilm, then incubated overnight at room temperature in a dark humid chamber. Coverslips were removed and slides were washed three times for 15 minutes in 0.1x SSC, dried, and then mounted in VECTASHIELD with DAPI (Vector Labs). Samples were imaged using a Leica Stellaris 8 confocal microscope with 63x oil-immersion objectives and processed using Fiji (ImageJ) software. Probes used for this study are as follows: 359, 5'-AGGATTTAGGGAAATTAATTTTTGGATCAATTTTCGCATTTTTTGTAAG-3'-Cy5; $(TAGA)_6$-Cy5; IGS, 5'-AGTGAAAAATGTTGAAATATTCCCATATTCTCTAAGTATTATAGAGAAAAGCCATTTTAGTGAATGGA-3'-Alexa488; $(AATAC)_6$-Cy3; and $(AATAAAC)_6$-Cy3.

Generation of rDNA deletion animals

rDNA copy number loss was induced during larval development in *yw* / Y ;; *HS-I-CreI*, *Sb* / TM6B males with a *y, w* X chromosome by I-CreI expression as previously described(*26*). In brief, parental animals mated and laid eggs for three days, then removed from food. After one additional

day of larval development, animals were exposed to 37° C heat shock for 45 minutes on two consecutive days. identify X chromosomes with significant rDNA copy number reduction (bb), adult males that experienced I-CreI expression were mated to *bb*^{158} / *FM6* females, and virgin non-*FM6* daughters (bb / bb^{158}) were screened for the *bobbed* phenotype. 28 out of 946 non-*FM6* daughters screened were *bobbed*. To isolate potentially reduced rDNA loci and remove HS-I-CreI from the background, TM6B containing *bobbed* females were individually mated to wild-type males. Male offspring from each individual female candidates were subsequently individually mated to *bb*^{158} / *FM6* females. Any mating that failed to produce non-FM6 daughters were eliminated (due to having the bb^{158} and not the candidate bb chromosome). All viable non-FM6 daughters were double checked for the *bobbed* phenotype, and stocks with all *bobbed* non-FM6 daughters had FM6 containing siblings collected and used to establish a bb / FM6 stock. This method isolated the novel rDNA deletion allele, bb^{Z9}, used in this study.

Drosophila genetics

All Drosophila lines used in this study are found in **Table S2**. All animals we reared on standard Bloomington medium at 25º. All aging was done in roughly 1:1 mixed presence of males and females, provided fresh food every four to six days. UAS:R2 RNAi strains were designed using SNAP-DRAGON shRNA target software, and oligos containing target hairpin sequence were cloned into the WALLIUM20 vector for phiC31 site-directed integration into the Drosophila genome. All UAS:R2 transgenes were synthesized into pUAST:attB by VetcorBuilder (Chicago, IL) for site-directed integration. All injections and selection of animals containing integrated transgenes were performed by BestGene, Inc (Chino Hills, CA).

Lineage survival assay

Independent lineages of nos:Gal4 / CyO; UAS:R2 RNAi / Tm6B or nos:Gal4 / CyO; TM2 / TM6B animals were established by collecting siblings of the indicated genotypes from nos:Gal4 / CyO;

TM2 / TM6B males mated to Sp / CyO; UAS:R2 RNAi / TM6B females. At each generation in each lineage, 3 males were mated with 5 females for 5 days, and offspring were collected 10 days after mated ended. Any lineages that did not have at least 3 males and 5 females at collection time were terminated due to insufficient animals.

Statistics

For all comparisons of percentage of samples with categorical values (percent γH2Av or R2 positive cells), significance was determined by chi-squared test. For all comparisons of samples with independent values (number of GSCs; rDNA copy number), significance was determined by Student's t-test between experimental and control conditions, unless otherwise indicated.

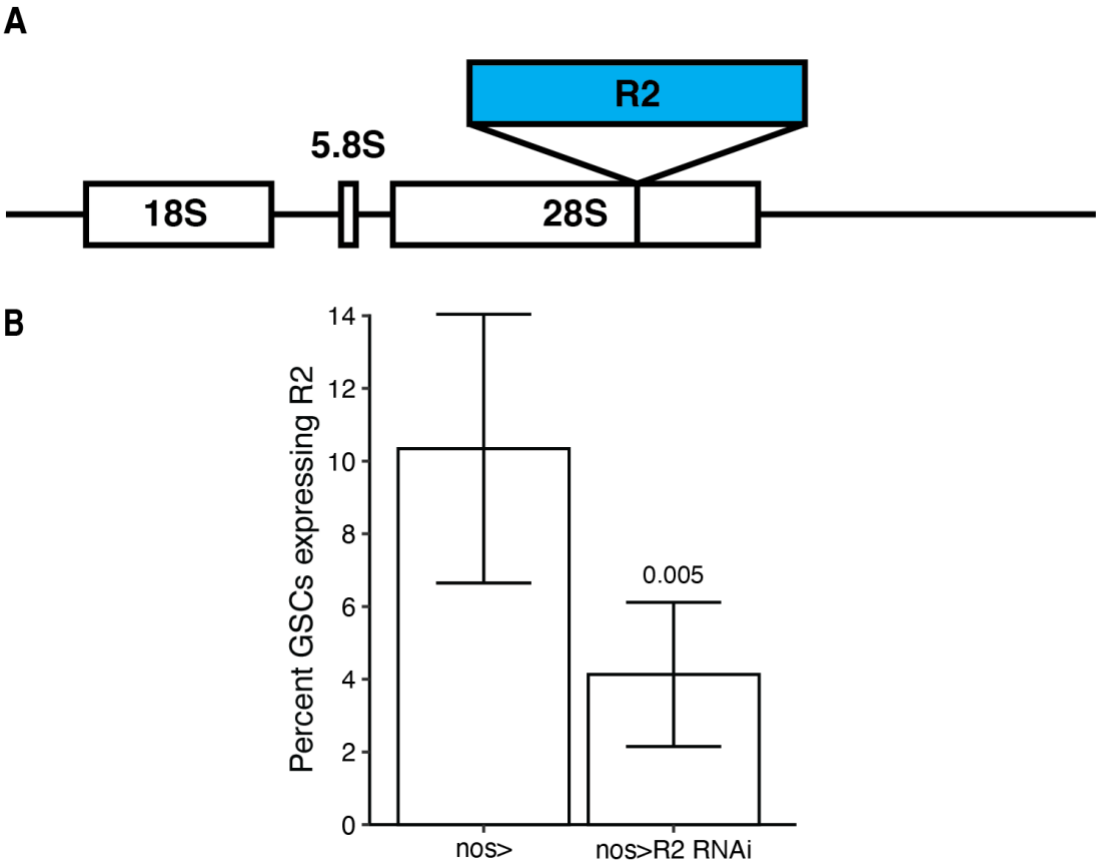

Figure S1: Design and efficiency of R2 RNAi. (A) Diagram the 45S rRNA cistron constituting an 'rDNA copy' and the sequence-specific R2 insertion site in the 28S rRNA gene. **(B)** Confirmation of R2 expression knockdown by R2 RNAi. Precent of GSCs expressing R2 detected by RNA FISH in *nos-gal4* driver only condition (nos>) and R2 RNAi expressed by the *nos-gal4* driver (nos>R2 RNAi). P-value determined by chi-squared test. Error bars = 95% CI.

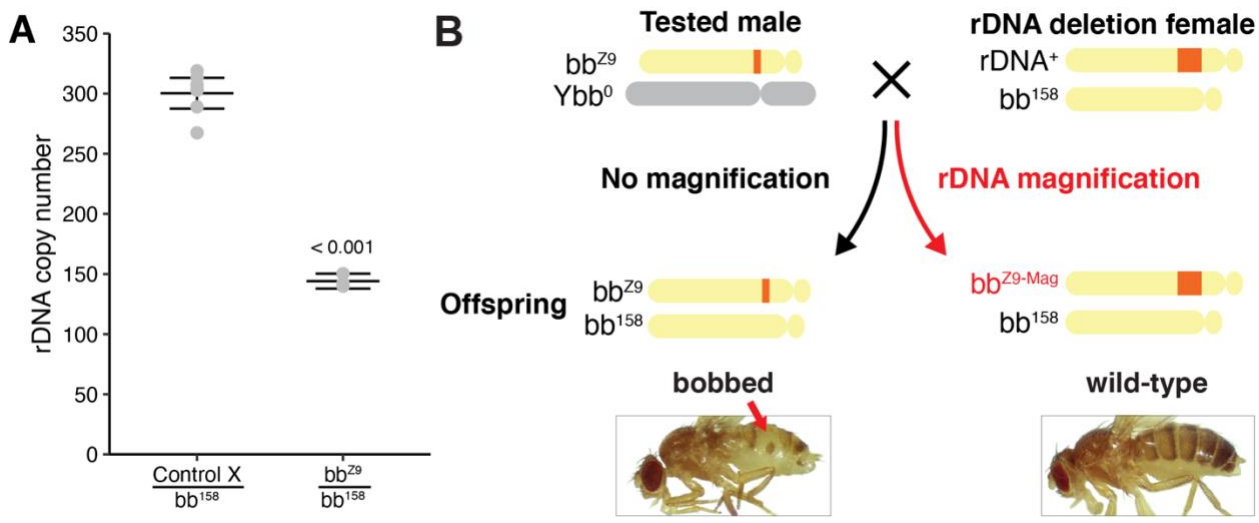

Figure S2: Diagram of phenotypic assessment to detect rDNA magnification at the bb^{Z9} allele. (A) rDNA CN quantification of the bb^{Z9} allele determined by ddPCR. All samples from individual females with either the bb^{Z9} allele or X chromosome from which the allele was isolated heterozygous with an X chromosome completely lacking rDNA (bb^{158}). P-value determined by Student's t-test. Error bars = 95% CI. **(B)** Scheme to detect rDNA magnification of bb^{Z9} allele. Tested magnifying males (bb^{Z9} / Ybb^{0}) are crossed to females heterozygous for the bb^{158} rDNA deletion allele. Resultant bb^{Z9} / bb^{158} daughters completely rely on the bb^{Z9} locus for their rDNA. (Left) bb^{Z9} / bb^{158} offspring with the 'bobbed' cuticular defects associated with rDNA insufficiency (red arrow) are determined to have inherited a bb^{Z9} allele that did not undergo rDNA magnification. (Right) bb^{Z9} / bb^{158} offspring with wild-type cuticles are determined to have inherited a bb^{Z9} allele that restored normal rDNA content through rDNA magnification ($bb^{Z9\text{-}Mag}$). Thus, the portion of bb^{Z9} / bb^{158} offspring with wild-type cuticles represents the portion of bb^{Z9} alleles that underwent rDNA magnification. bb^{Z9} / bb^{158} animals are also collected for rDNA CN quantification, and total rDNA CN in these animals represents the rDNA from the bb^{Z9} locus alone.

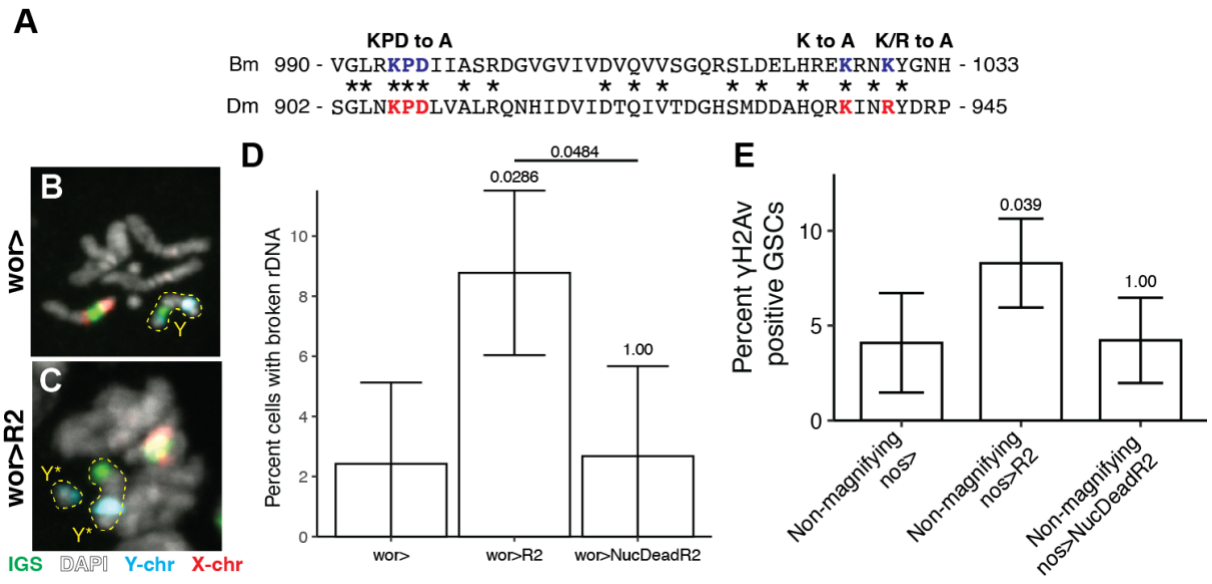

Figure S3: Transgenic R2 expression induces double stranded breaks at rDNA loci. (A) Alignment of 44 amino acids in the endonuclease domain of *Bombyx mori* R2 (Bm) and *Drosophila melanogaster* R2 (Dm). Residues shown to be essential for Bm R2 endonuclease activity(*23*) labeled in blue and respective Dm R2 residues in red. Mutations found to render Bm R2 endonuclease dead(*23*) and their respective amino acid changes in Dm R2 are indicated above highlighted residues. **(B-C)** DNA FISH in mitotic chromosome spreads of larval neuroblasts in animals with the neuroblast driver *wor-gal4* (wor>, B) or transgenic R2 expressed by the *wor-gal4* driver (wor>R2, C) animals. rDNA is labelled by probes targeting the rDNA intergenic sequence (IGS) (Green), Y- chromosome is labelled by probes targeting AATAC and AATAAAC satellite sequences (Cyan) and X-chromosome is labelled by probes against 359 and TAGA satellite sequences (Red). Y* indicates Y-chromosome fragments broken at the rDNA locus. **(D)** Percentage of neuroblasts with DSBs at rDNA loci determined by DNA FISH in mitotic spreads in indicated genotypes. P-values determined by chi-squared test. Error bars = 95% CI. **(E)** Percentage of γH2Av positive GSCs in the indicated genotypes. Transgenic R2 constructs expressed in the germline by the *nos-gal4* driver (nos>). P-value determined by chi-squared test. Error bars = 95% CI.

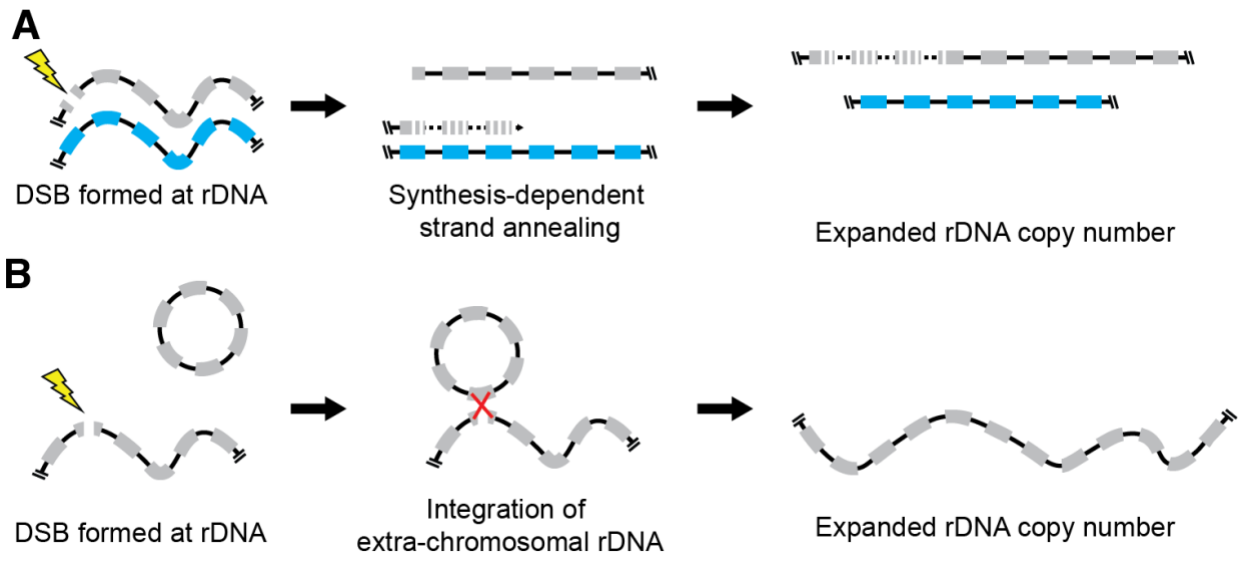

Figure S4: Models of rDNA CN expansion initiated by DSBs at rDNA locus. (A) rDNA CN expansion by strand-dependent strand annealing (SDSA). DSBs at rDNA loci can be repaired via recombination of a single strand with sister chromatid and synthesis. Synthesis can run on to replicate multiple rDNA copies before re-annealing with broken strand. Upon repair after synthesis, rDNA CN is expanded on one sister chromatid while CN of the other sister remains intact. **(B)** DSBs formed at rDNA loci induce homology-dependent repair that causes recombination with existing extrachromosomal rDNA circles (ERCs), allowing for reintegration of the ERC rDNA copies into the genomic rDNA locus.

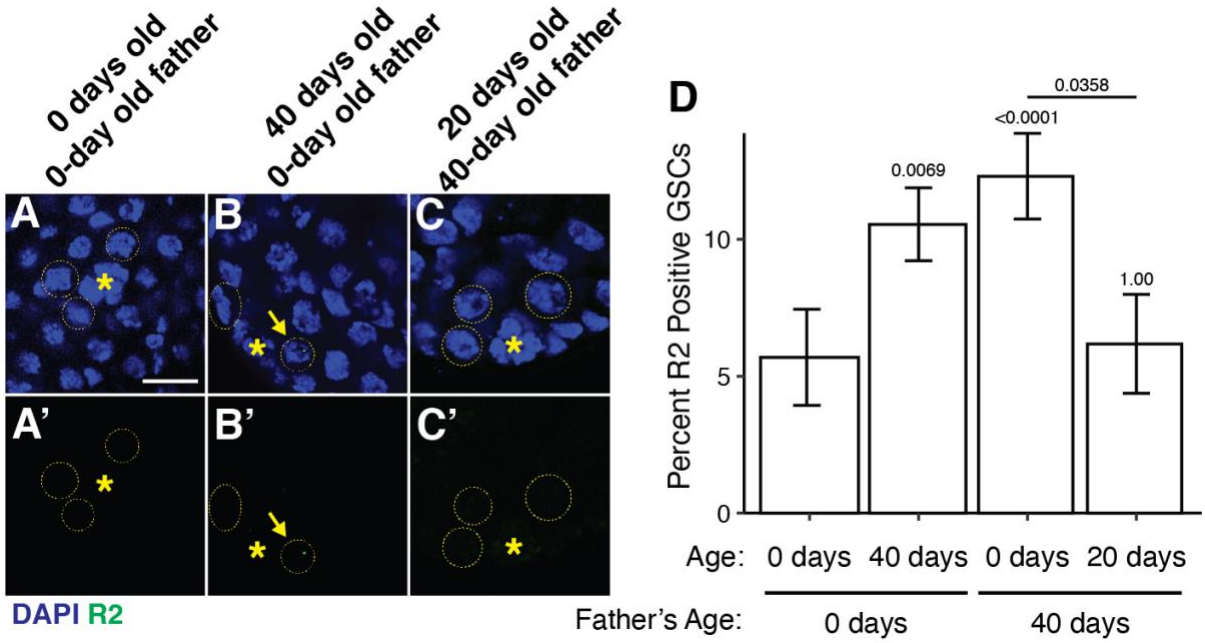

Figure S5: R2 expression in GSCs is regulated in response to changing rDNA copy number during aging. (A-C) R2 RNA FISH in the male germline of young (0-days old), (A) old (40-days old) (B), and recovered offspring of old animals (C). Isolated R2 channel in A'-C'. GSCs indicated by yellow dotted circle. * indicates GSC niche signal hub. R2 positive cells indicated by yellow arrowhead. **(D)** Percentage of R2 positive GSCs at indicated ages in the offspring of 0- or 40-day old fathers. P-value determined by chi-squared test. Error bars = 95% CI.

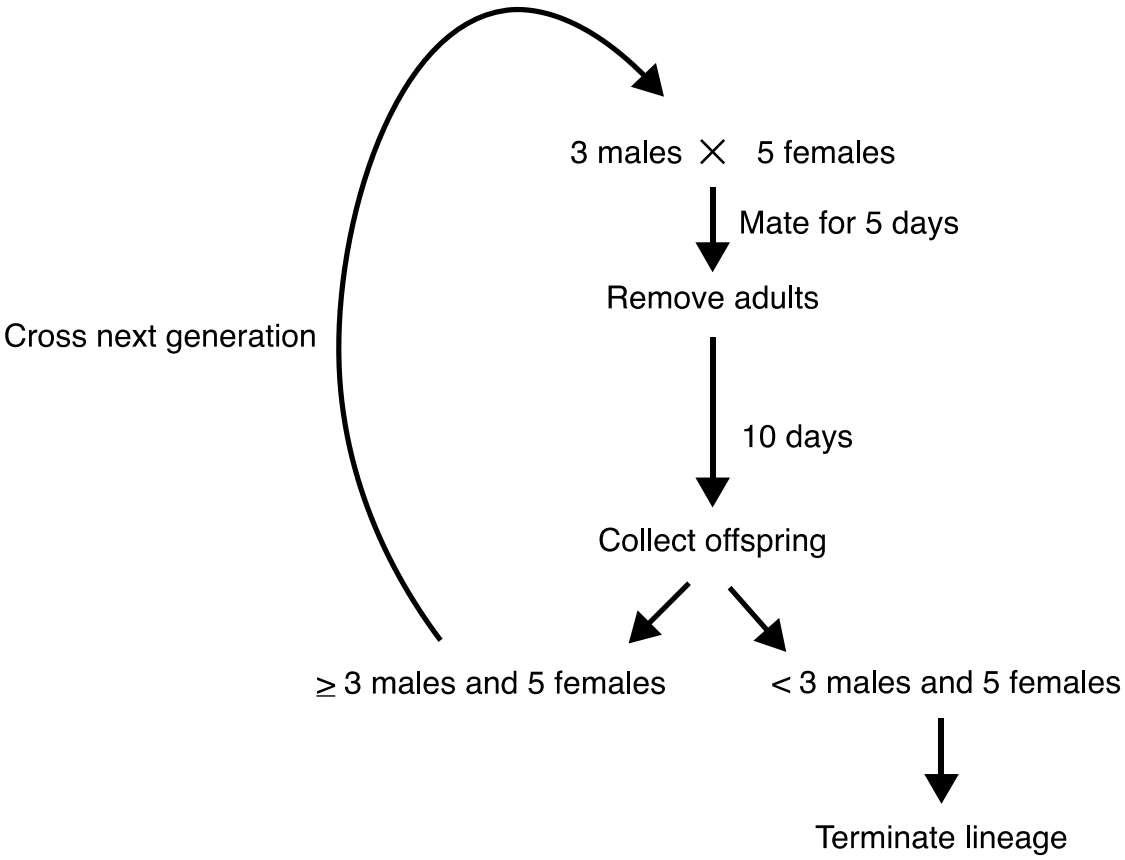

Figure S6: Scheme for testing lineage survival in multi-generational R2 RNAi experiment. Each lineage is established using 3 males and 5 females of the given genotype. Males and females are mated for 5 days and removed from the vial. 10 days later, all offspring are collected. If total offspring from the mating contains at least 3 males and 5 females, then 3 males and 5 females are randomly selected to establish the next generation of the lineage. If the total offspring contained fewer than 3 males or 5 females, then the lineage was terminated and future generations were not established. This procedure was repeated at each generation for each lineage for 6 generations or until termination.

Table S1: List of oligomers used in this study for ddPCR assays.

Oligo Name	Type	Target	Sequence	Modifications
dd-RpL32 F	Primer	RpL32	GCTTCAAGGGACAGTATCTG	
dd-RpL32 R	Primer	RpL32	AACGCGGTTCTGCATGAG	
dd-RpL32 Probe	Probe	RpL32	ATGCCCAACATCGGTTAC	5' HEX AND 3' Iowa Black FQ
dd-28S F	Primer	28S	GAGCTGCCATTGGTACAG	
dd-28S R	Primer	28S	GCTTTCGCCTTGAACTTAG	
dd-28S Probe	Probe	28S	TGGTGGATAGTAGCAAATAATCG	5' 6-FAM AND 3' Iowa Black FQ
dd-Upf1 F	Primer	Upf1	CACACTTTATGTCCACCATTATTG	
dd-Upf1 R	Primer	Upf1	GAGTTTCCGTAGGGACCAC	
dd-Upf1 Probe	Probe	Upf1	CCG TAA CCG CCA CTG CGG T	5' 6-FAM AND 3' Iowa Black FQ

Table S2: List of Drosophila stocks used in this study.

Genotype	Source	Use
Dp(1;f)122; C(1)RM, y^1 v^1 f^1 / C(1;Y)6, Df(1)259, w^1	Kyoto Drosophila Genomics and Genetic Resource Center (DGRC) #107274	Mini-x Chromosome (*Dp(1;f)*)
yw	Bloomington Stock Center (BSC) #1495	Source for isolating novel rDNA deletion strain
w[1118]; P{v[+t1.8]=hs-I-CreI.R}1A Sb[1]/TM6	BSC #6937	I-CreI endonucelase
nos-gal4	PMID: 9501989	Early germ cell driver
wor-gal4	BSC #56553	Neuroblast driver
bam-gal4	PMID: 12571107	Germ cell driver
y[1] eq[1]/Df(YS)bb[-]	DGRC #101260	Y chromosome complete rDNA deletion
Df(1)bb158, y[1] / Dp(1;Y)y[+] / C(1); ca[1] awd[K]*	DGRC #106876	X chromosome complete rDNA deletion

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