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TITLE:
Genetic Determinants Influencing the Maternal Transmission and Somatic Accumulation of Mitochondrial Mutations

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We are using the common fruit fly, *Drosophila melanogaster*, as a model organism to pursue two aims. The first aim is focused on using a novel, high accuracy, next-generation sequencing methodology to perform a genome wide screen for genes involved in selecting against the accumulation and/or transmission of mtDNA mutations. The second aim is focused on characterizing the effects of putative modifiers on mitochondrial bioenergetics and morphology in hopes of providing insight into how these modifiers function. During the current reporting period, we have determined that the exonuclease deficient Pol-gamma strains that we originally proposed to use for our screening studies are likely not suitable due to the fact that mutations accumulate in the eggs during oogenesis. To solve this problem, we have developed a mitochondrially targeted cytidine deaminase, APOBEC1, expressing fly strains that express this protein solely in the germline or solely in the somatic tissue. We have begun to characterize the flies expressing APOBEC1 in the somatic tissue. This strain shows a large increase in C→T/G→A mutations, as expected, as well as a significantly reduced lifespan and loss of climbing ability. We are beginning the characterization of the germline expressing strain. We will begin our screening efforts upon the completion of our characterizations.

15. SUBJECT TERMS
Duplex Sequencing, mitochondrial APOBEC1, germline mutations, somatic mutations.

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I. INTRODUCTION

The focus of the grant is on mitochondrial diseases. Owing to their evolutionary history, mitochondria have retained a small circular genome that encodes essential components of the electron transport chain, which provides the majority of the energy required for an organism to live. Mutations in the mitochondrial genome can cause the electron transport chains to become dysfunctional. These mutations can be potentially passed to the offspring of the female carrier or accumulate with advancing age. If these mutations reach a high enough level within a tissue, a number of devastating diseases can occur. Remarkably, there is significant evidence to suggest that mechanisms have evolved to prevent the transmission or accumulation of the most deleterious of these mutations. However, even in the presence of these selection mechanisms, diseases involving mtDNA mutations occur. The goal of the funding is to understand the mechanism(s) that are involved in preventing the accumulation of these mutations either through the germline or during development and aging of the somatic tissue by identifying candidate genes involved in this process. To begin to identify these mechanisms, we are using the common fruit fly, *Drosophila melanogaster*, as a model organism to pursue two aims. The first aim will focus on using a novel, high accuracy, next-generation sequencing methodology to perform a genome wide screen for genes involved in selecting against the accumulation and/or transmission of mtDNA mutations. The second aim will focus on characterizing the effects of putative modifiers on mitochondrial bioenergetics and morphology in hopes of providing insight into how these modifiers function.

II. KEYWORDS

Mitochondrial DNA, Duplex Sequencing, Somatic Mutations, Mitochondrial Disease,

III. ACCOMPLISHMENTS

A. What are the major goals of the project?

The project encompasses three major goals.

1. Perform genetic crosses.
   Subtask 1: Acquire and establish fly deletion strains from the Bloomington Stock Center.

   Subtask 2: Perform crosses between the fly deletion strains and the transgenic DNA polymerase gamma mutator strain.

   Subtask 3: Microdissect fly brain and ovaries. Purify DNA for sequencing (Major Task 2).

   Subtask 4: Monitor health parameters, such as lifespan and climbing index, to look for suppressors of high mutations loads.

2. Sequence mtDNA using Duplex Sequencing to measure the mutation frequency of mtDNA in the *Drosophila* germline.

   Subtask 1: Sequence the ND2 gene of the mtDNA purified from the ovary of strains created during the initial crosses with the highly accurate Duplex Sequencing methodology.

   Subtask 2: Use RNAi and available knockout fly strains to isolate and identify the specific gene responsible for the increased transmission of mutations in the germline—
   Subtask 3: Confirm candidate modifiers by sequencing the entire mitochondrial genome by Duplex Sequencing.

3. Characterize the biochemical and cellular phenotypes.

   Subtask 1: Determine specificity of candidate genes for the germline vs the soma by comparing mutation load of ovary to isolated brain.

   Subtask 2: Measure biochemical and cellular parameters that may be involved in mitochondrial selection. This will include determining effects on mitochondrial morphology, mitochondrial activity, functional staining, *etc* in the ovary.

B. What was accomplished under these goals?

In the last reporting period, we indicated that we would develop an alternative approach to using a Pol-γ^{exo-} mutator strain to induce and study germline mtDNA mutations. Specifically, we settled on targeting the cytidine deaminase APOBEC1, which causes a very specific G→A/C→T mutation type, to the mitochondria. We previously showed that the APOBEC1 transgene being driven in the somatic tissue by the pan somatic *daughterless* driver accumulated large numbers of mutations consistent with APOBEC1 expression. This work was recently submitted to *Nature Communications*.

To induce specific mtDNA mutations in the germ-line, we took a two pronged approach. First, as indicated in the previous reporting period, we designed a fly that expresses APOBEC1 under the control of the germ stem cell specific *bgcn* promoter, which is only expressed in the germ stem cell prior to the germline genetic bottleneck is predicted to occur (oogenesis Stage IIb). We obtained viable strains and sequenced mtDNA purified from dissected brain tissue of their progeny. Unfortunately, no detectable changes in mtDNA mutation frequency or type were observed in the offspring, suggesting that this approach either does not express properly or do does not induce enough mutations to be seen by Duplex Sequencing.

In our second approach, Cyo/UAS-APOBEC1 flies were crossed with a strain of flies that expresses the transcription factor that stimulates the UAS sequence, Gal4, under the control of three different ovarian drivers (MTD): the Bloomington Drosophila Stock Center ID 31777 P{w[+mC]=otu-GAL4::VP16.R}1, w[*]; P{w[+mC]=GAL4-nos.NGT}40; P{w[+mC]=GAL4::VP16nos.UTR} CG6325[MVD1] (Fig.1). *otu* and *nos* drivers have previously been reported to be expressed in early oogenesis (Fig.1). Since both stocks (UAS-APOBEC-1 and 31777) were found in homozygous dose, the first progeny produced from these crosses (F1) would be expected to be heterozygous for both constructs (Fig. 2) and therefore, expressing Gal4 in early stages of oogenesis of the F1 females and consequently stimulating the expression of APOBEC-1.

To test whether the heterozygous F1 females were expressing APOBEC and inducing mtDNA mutations in their germ-line as expected, the rate of low level mtDNA mutations was assessed by performing mtDNA Duplex Sequencing of the F1 heterozygous females and their F2 progeny. An increase in the frequency and number of mtDNA mutations and a predominance of C-to-T transitions was expected to be observed in the F2 progeny compared to the F1 mothers. DNA was extracted from the fly's heads and the detected mtDNA mutations were compared between the mother and their F2 daughters. A fly strain expressing the same UAS-APOBEC-1 construct but with a mutation that disrupt the deaminase region (E63A) and therefore expresses a catalytically dead APOBEC-1 protein was used as a control. To eliminate the possibility of undesired UAS-APOBEC-1 or 31777 expression in the F2 progeny because of inheriting one of these constructs from the F1 mother, F1 virgins were crossed to Cyo/Gla males. The F1 progeny was phenotypically heterogeneous but only flies with no eye color were selected for DS, since they were not expressing any of the transgenes (APOBEC or MTD drivers).

DS revealed a significantly increased frequency of point mutations in the F1 mothers and the F2 w daughters (Fig. 3A) with G-A/C-T mutations as the predominant point mutation (Fig. 3C). Bioinformatic analysis of the sequence surrounding these G-A/C-T mutations were consistent with the APOBEC-1 mutation signature described in cancer (Fig.4). This difference was not seeing in F1 or F2 flies with the catalytically dead APOBEC-1 (Fig.5). The increased frequency of mutations in the F1 progenitor suggests that both MTD-driven Gal4 and UAS_APOBEC-1 are being expressed very early in the embryogenesis of the F1 female progenitor and, because mutations are detected in the fly head (somatic tissue), these mutations are being expanded clonally as the fly developed and reached adulthood. This observation was confirmed by performing a replicate study with 3 additional independently obtained F1 females and this time including the original progenitor (PO) to exclude the possibility that the observed mutations were inherited.

In a replication study, heterozygous F1 female virgins were crossed to w1118 males (the same genetic background of both stocks) instead of Cyo/Gla males and the rate of mtDNA mutations in the F1 mother and F2 daughters was performed as the initial study. By using w1118 males, this replication study eliminated phenotypic confounders that were associated with the Cyo/Gla background. In both the initial and the replication studies, the F2 progeny was also found to have an increased frequency of point mutations although in the initial study the frequency of point mutations in the F2 progeny is lower than in the replication. Since the F1 female flies are expected to express both transgenes in their germ-line, the mutations detected in F2 flies could be the result of both inheritance of mtDNA mutations from F1 mothers and induction of new mtDNA mutations by APOBEC during oogenesis. Interestingly, the clonality of each individual point mutation was found to increase from F1 to F2 females in both the initial and the replication studies (Fig.6) while mutations in the F1 females did not exceed clonality of 0.1 many mutations detected in F2 females were above or double this clonality. This observation suggests that

mtDNA point mutations detected by DS in this study are the result of a combination of mutagenesis in the early embryogenesis of the F1 female and in early cells in their germarium, the increased clonality of the mutations detected in F2 females may be the result of clonal expansion of mutations that escape the mitochondrial bottleneck in the F1 germarium and become part of the become more clonal in the early in F1 oogenesis.

Dilution of APOBEC-induced mtDNA mutations and clonal expansion of mtDNA mutations through multiple generations

After successfully being able to detect low level point mtDNA mutations in the F1 and F2 somatic tissue, F2 females were further crossed to background males and the mtDNA mutations were followed up by 3 more generations. The frequency of mtDNA point mutations dropped gradually with each generation whereas the clonality of these mutations increased (Fig.7).

Characterization of APOBEC-1 induced mtDNA mutations

The sequencing data was further analyzed to identify specific mutations inherited from the F1 female and past down in each generation. More detailed examination of specific G-A/C-T mutations inherited through 4 generations revealed different patterns of frequency and clonality. First, mutations that were shared from F1 to F4 progeny were considered to have originated during F1 embryogenesis. These shared mutations were observed to have increasing clonality from F1 to F3 which decreases in F4 progeny in most of the lineages (Fig.8). This pattern was similar between synonymous and nonsynonymous mutations.

Second, mutations that were shared between F2 and their progeny and that were not detected in F1 females were considered to have originated in the germarium of F1 females and were expected to have high frequency in F2 adults and increased clonality in F3 and F4 progeny. These group of mutations had a similar clonality of that of the "F1-originated"mutations and their clonality in subsequent generations was variable with no tendency to increase in F3 (Fig.9).

If both synonymous and nonsynonymous mutations are inherited with higher clonality from F1 to F2 flies it is possible that mitochondrial quality control systems are not selecting against any of those mutations and it is therefore possible that the actual clonality and frequency of APOBEC-induced mutations is higher than it is observed in adult tissue because non-viable eggs or eggs carrying lethal mutations are not developing to adulthood. An estimation of the clonality of synonymous and nonsynonymous mutations in one single egg from an F1 fly revealed that the amount of different nonsynonymous mutations in one egg is much higher than the one seen in an F2 fly (Fig.10)

To further study if mtDNA mutations were differentially inherited based on their type, a separation of synonymous and nonsynonymous mutations based on whether or not they were inherited to the next generation was performed and their clonality compared (Fig. 11). Among the inherited mutations (Fig.11A), synonymous mutations (Fig. 11A, *left*) were more clonal than non-synonymous (Fig. 11A, *right*). Mutations that were not inherited to the next generation were, in general, less clonal than the inherited mutations (Fig. 11B) and showed no difference in their clonality levels. A comparison of the amount of synonymous and nonsynonymous mutations being inherited or not (Fig. 12) revealed that there is a higher amount of synonymous mtDNA mutations in the F1 fly (ratio S/N= 3.3). this ratio slightly changes for the mutations that were inherited by the F2 progeny (0.4 vs. 0.3) because the proportion of mutations by the F2 was the opposite of that seen in the F1: much higher amount of nonsynonymous mutations in the F2, which was much higher for the non-inherited than for the inherited mutations. This pattern is maintained in the F3 and F4 generations.

Lastly, F1 and F2 flies were studied for their lifespan by performing aging assessment in 10 flies of each genotype. No significant differences are seen in F1 and F2 flies when comparing flies expressing the UAS-APOBEC-1 wild type with flies expressing the catalytically dead APOBEC (Fig.13). Ongoing studies are being performed to test F3 and F4 generation flies.

C. What opportunities for training and professional development has the project provided?

Nothing to Report

D. How were the results disseminated to communities of interest?

Nothing to Report

E. What do you plan to do during the next reporting period to accomplish the goals?

We will begin to perform our intended screening of genes as outlined in our original proposal.

IV. IMPACT

A. What was the impact on the development of the principal discipline(s) of the project?

We have developed two new genetic systems to better explore mitochondrial mutations in both the germline and somatic tissues. These include making *Drosophila* strains that express the cytidine deaminase APOBEC1 under the expression of the UAS-Gal4 driver system and under the germline specific *bgcn* promoter, which is only expressed in the germ stem cell. These are novel creations and will be important tools going forward.

B. What was the impact on other disciplines?

Nothing to Report

C. What was the impact on technology transfer?

Nothing to Report

D. What was the impact on society beyond science and technology?

Nothing to Report

V. CHANGES/PROBLEMS

A. Changes in approach and reasons for change.

In the last reporting period, we proposed to develop a new genetic system to deal with this problem by separating the accumulation of somatic mutations from the accumulation of the germline mutations by expressing APOBEC1 under the germline specific *bgcn* promoter. We were unable to induce mutations with this approach, but we were able to salvage our germline specific APOBEC1 expression by driving with the germline specific Maternal Triple Driver strain. This approach gave robust induction of germline mutations. Because of the significant change in the experimental system, we have had to spend time characterizing the phenotypes and new system. The objectives of the proposal remain the same.

B. Actual or anticipated problems or delays and actions or plans to resolve them.

The second issue we encountered deals with the genetic system we originally proposed to work with in our application. As mentioned in Section IIIB, we had designed an experimental system to express APOBEC1 under the *bgcn* promoter, which only expresses in the germ stem cell. We engineered this transgenic fly, but were unable to observe mutations, even with evidence of gene expression.

To solve this issue, we engineered two *Drosophila* strains that express the cytidine deaminase APOBEC1 targeted to the mitochondria under with different drivers. This enzyme induces C→T mutations. To express APOBEC1 in the somatic tissue, we used the standard UAS-Gal4 driver system with Gal4 being driven by the pan somatic *daughterless* (*da*) promoter. Importantly the UAS-*da*Gal4 does not express in the germline, thus preventing mutations from accumulating in the germline. Separately, to specifically induce C→T mutations in the germline mtDNA, we designed a fly that expresses APOBEC1 under the control of the germ stem cell specific *bgcn* promoter, which is only expressed in the germ stem cell prior to the germline genetic bottleneck is predicted to occur (oogenesis Stage IIb). The *bgcn* promoter is not expressed at any other time during oogenesis. This effectively isolates the accumulation of germline mutations to the germ stem cell and reduces the potential confounders that arise from maternal transfer of DNA polymerase gamma mRNA.

C. Changes that had significant impact on expenditures.

Nothing to Report

D. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Not Applicable

E. Significant changes in use or care of human subjects.
Not Applicable

F. Significant changes in use or care of vertebrate animals.
Not Applicable

G. Significant changes in use of biohazards and/or select agents
Not Applicable

VI. PRODUCTS

A. Publications, Conference Papers, and Presentations

Two papers are currently in revision:

1) **Deleterious mitochondrial DNA point mutations are overrepresented in *Drosophila* expressing a proofreading-defective DNA polymerase γ**

   Colby L Samstag, Jake G Hoekstra, Chiu-Hui Huang, Mark J Chaisso, Richard J Youle, Scott R Kennedy*, Leo J Pallanck*
   Under revision at PLoS Genetics
   * Co-senior author

2) **A new mitochondrial DNA mutator model shows that quality not quantity of mutations affects organismal fitness in *Drosophila***
   Simonetta Andreazza, Colby L. Samstag, Alvaro Sanchez-Martinez, Erika Fernandez-Vizarra, Aurora Gomez-Duran, Juliette J. Lee, Roberta Tufi, Michael J. Hipp, Elizabeth K. Schmidt, Thomas J. Nicholls, Patrick F. Chinnery, Michal Minczuk, Leo J. Pallanck, Scott R. Kennedy, Alexander J. Whitworth
   Under revision at Nature Communications

One talk was given:
Absence of oxidative DNA damage-induced somatic mutations in mtDNA in aging and neurodegeneration
2017 American Society of Human Genetics annual meeting. Orange County Convention Center, Orlando FL

B. Website(s) or Internet Site(s)

Nothing to Report

C. Technologies or Techniques

Nothing to Report

D. Inventions, Patent Applications, and/or License

Nothing to Report

E. Other Products

Nothing to report

VII. PARTICIPANTS & COLLABORATING ORGANIZATIONS

A. What individuals have worked on this project?

Name:	Monica Sanchez-Contreras
Project Role:	Post-doctoral Fellow
Research Identifier:	ORCID 0000-0002-3092-2781
Nearest Person Month Worked:	11 calendar months
Contribution to Project:	Dr. Sanchez-Contreras performed the majority of experiments conducted so far. She optimized assay development, performed sequencing

	experiments, and has engineered our new transgenic *Drosophila* strain.
Funding Support:	CurePSP Foundation

Name:	Scott R Kennedy
Project Role:	Principal Investigator
Research Identifier:	ORCID 0000-0002-4444-1145
Nearest Person Month Worked:	1 calendar month
Contribution to Project:	Dr. Kennedy oversaw all experiments, helped analyze data, and administrated the grant.
Funding Support:	Department of Defense Rapid Innovation Fund National Institutes of Health R01 CurePSP Foundation National Institute of Justice CDMRP Lung Cancer Research Program

B. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Kennedy was awarded a grant from the National Institute of Justice. No changes to effort.

Dr. Kennedy was awarded a grant from the Department of Defense Congressionally Directed Medical Research Program Lung Cancer Research Program. No change in effort.

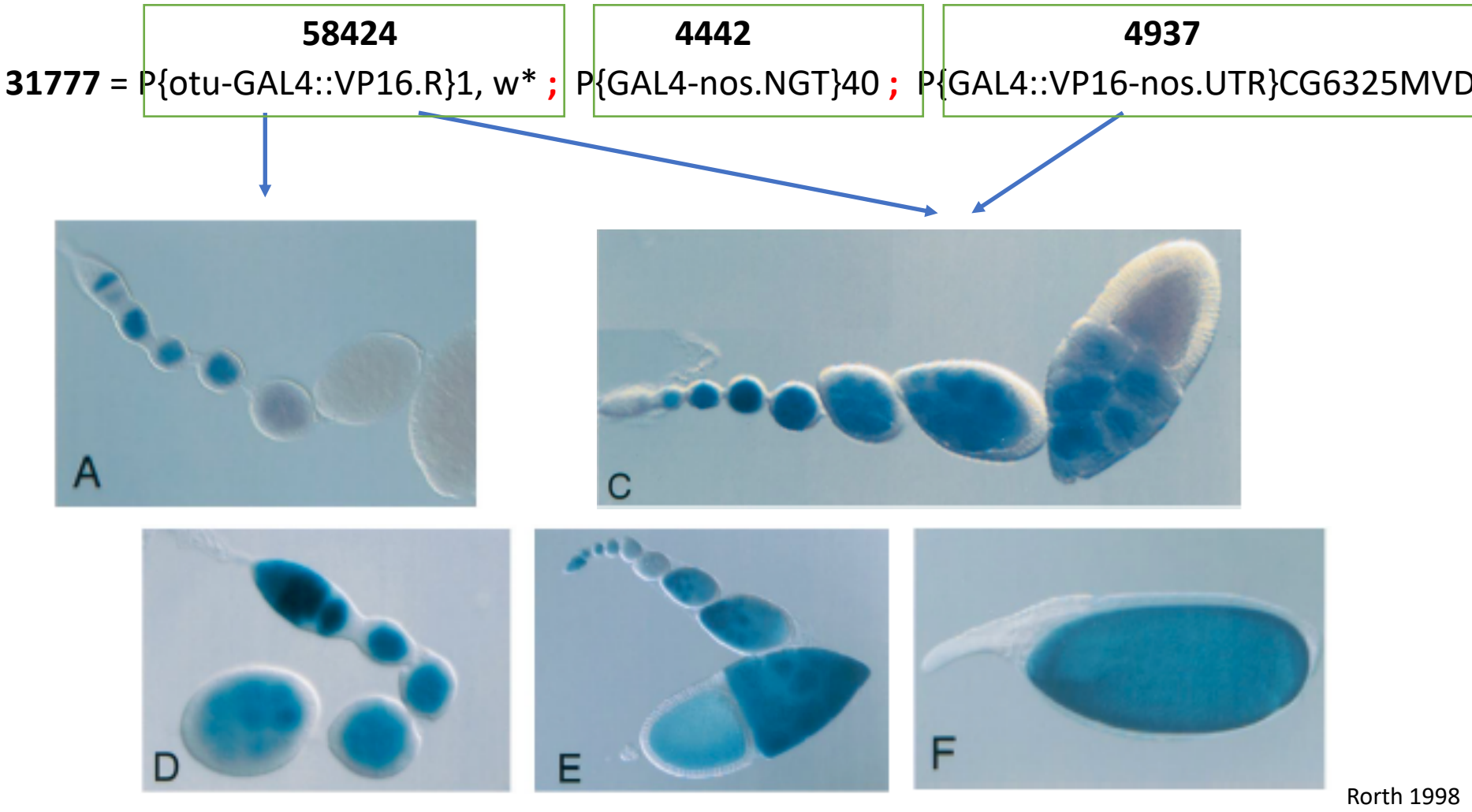
Dr. Kennedy acquired a one year grant from the CurePSP foundation. No changes to effort.

C. What other organizations were involved as partners?

Nothing to Report

VIII. SPECIAL REPORTING REQUIREMENTS

Nothing to Report



Rorth 1998

Figure 1: UAS system that only expresses Gal4 during oogenesis: MTD. Blue shows expression of beta-lactamase during different stages of oogenesis as driven by the indicated driver.

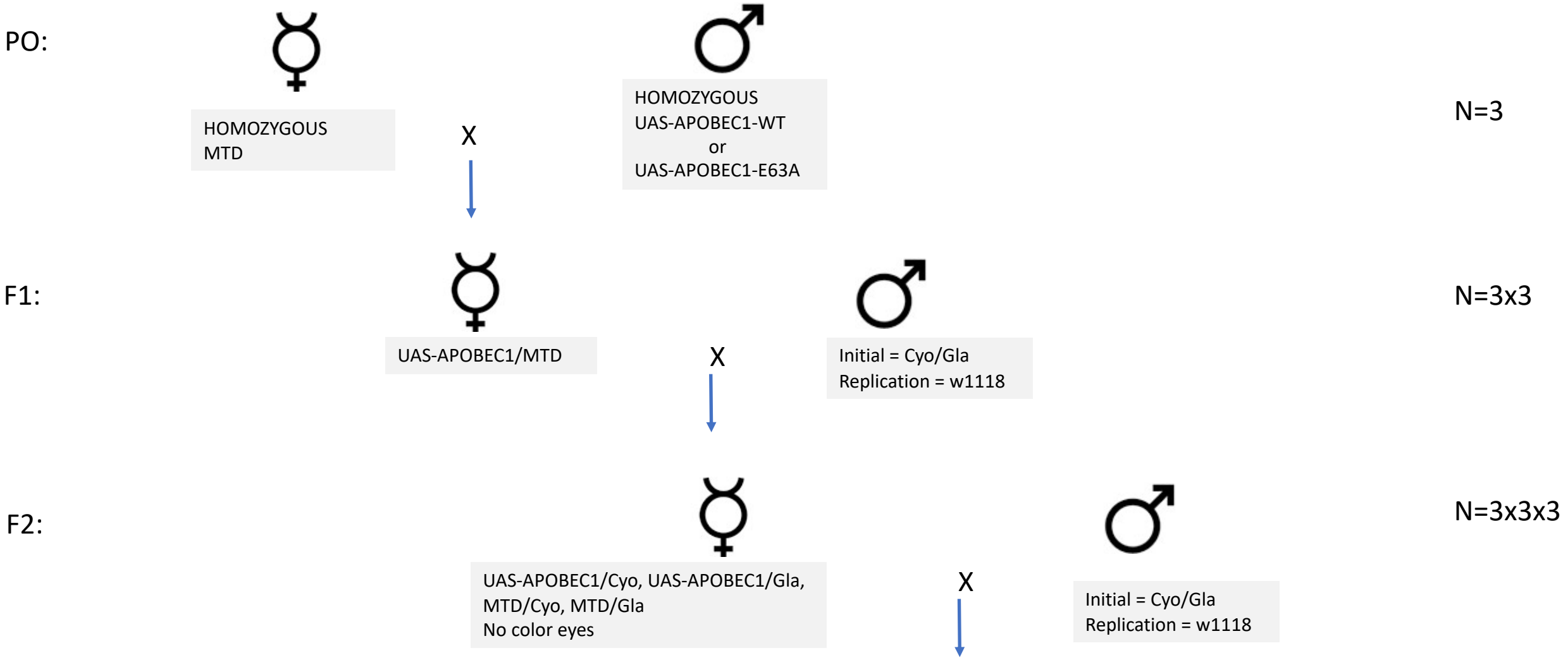


Figure 2: Fly crossing scheme used. MTD= BDS 31777 P{otu-GAL4::VP16.R}1, w*; P{GAL4-nos.NGT}40; P{GAL4::VP16-nos.UTR}CG6325MVD1

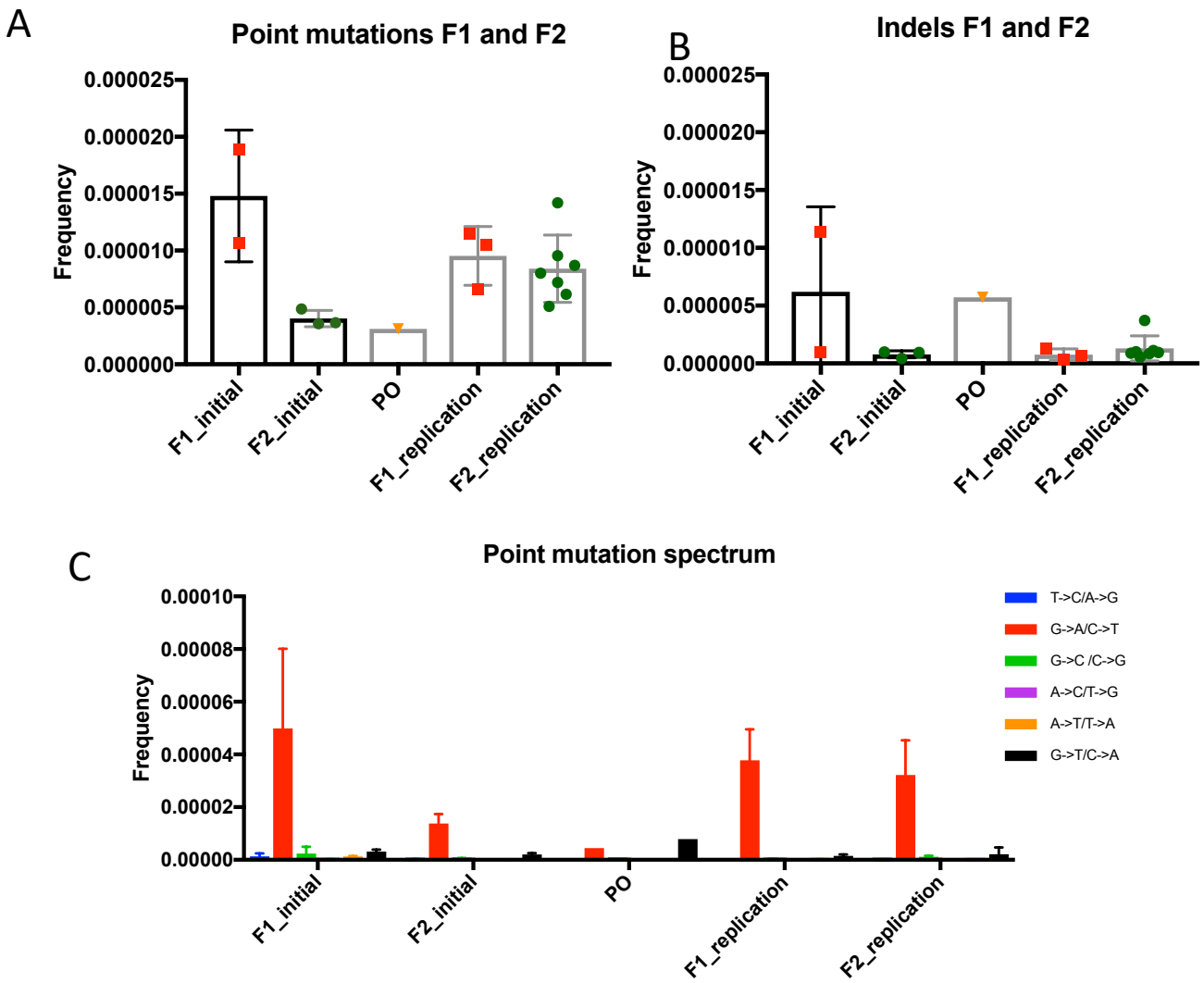
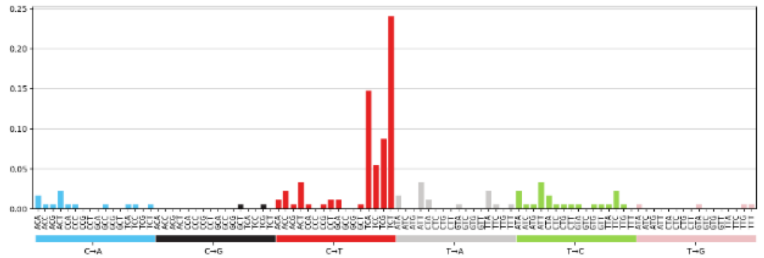


Figure 3: Point mutations induced by germline APOBEC1 across multiple generations. (A) Point mutation frequency. (B) Insertion and deletion frequencies do not significantly change across generations. (C) Mutation spectrum across two generations.

A

APOBEC1_WT



APOBEC1_E63A

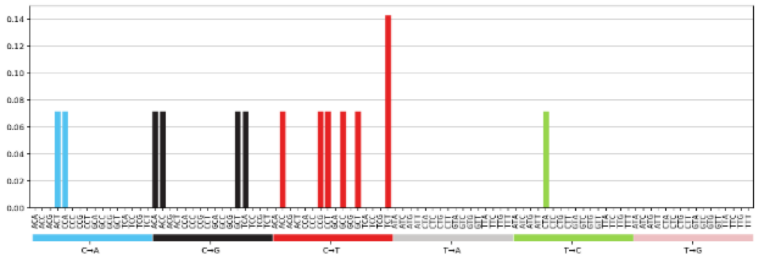


Figure 4: Frequency of low level mtDNA point mutations found in UAS-APOBEC/MTD F1 and F2 flies and their classification by type. Sequence signature of (A) APOBEC-1-induced mtDNA mutations, which is absent in (B) catalytically dead APOBEC1.

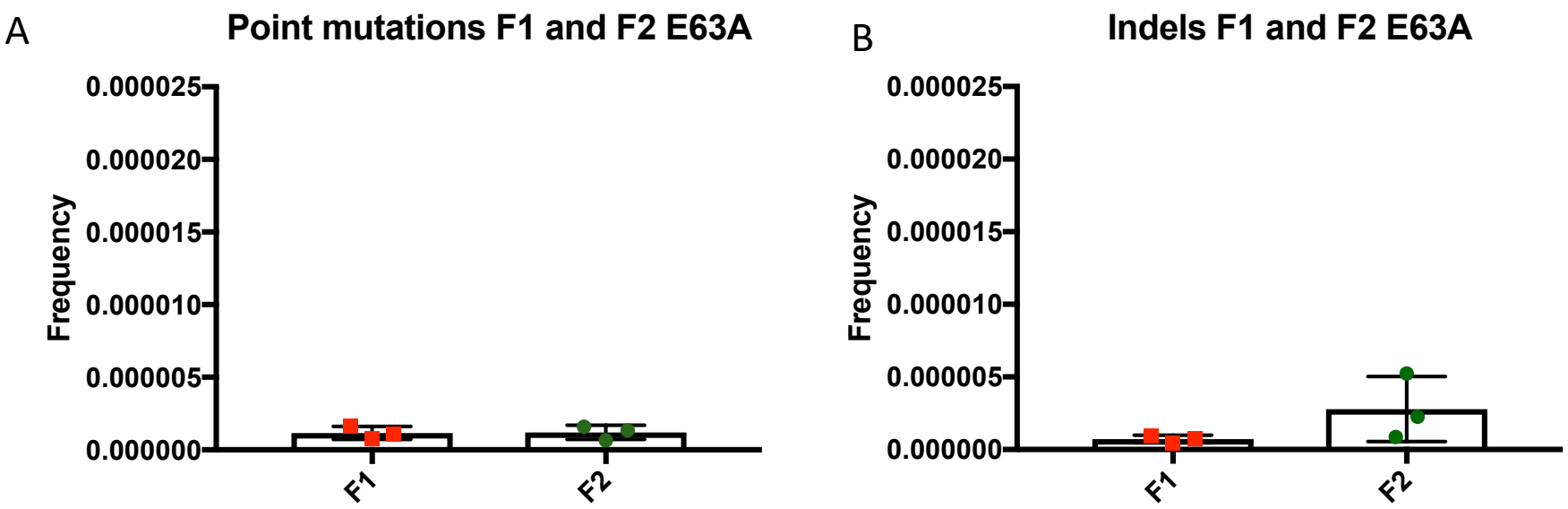


Figure 5: Mutation frequency of germline expressed catalytically deaAPOBEC1 across multiple generations. (A) Point mutation frequency. (B) Insertion and deletion frequencies do not significantly change across generations.

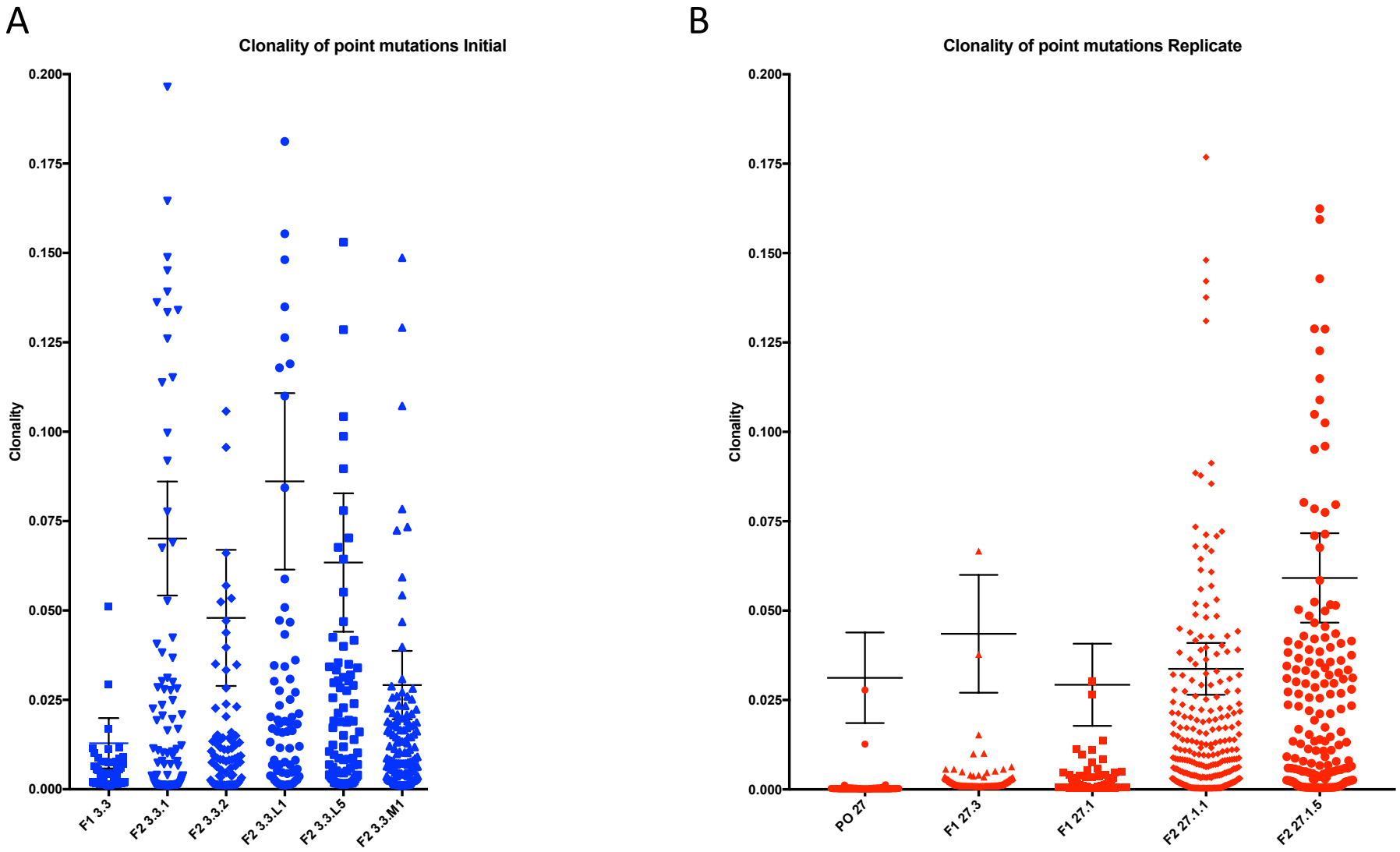


Figure 6: Point mutation clonality increases across multiple generations. (A) and (B) are from two independent lineages

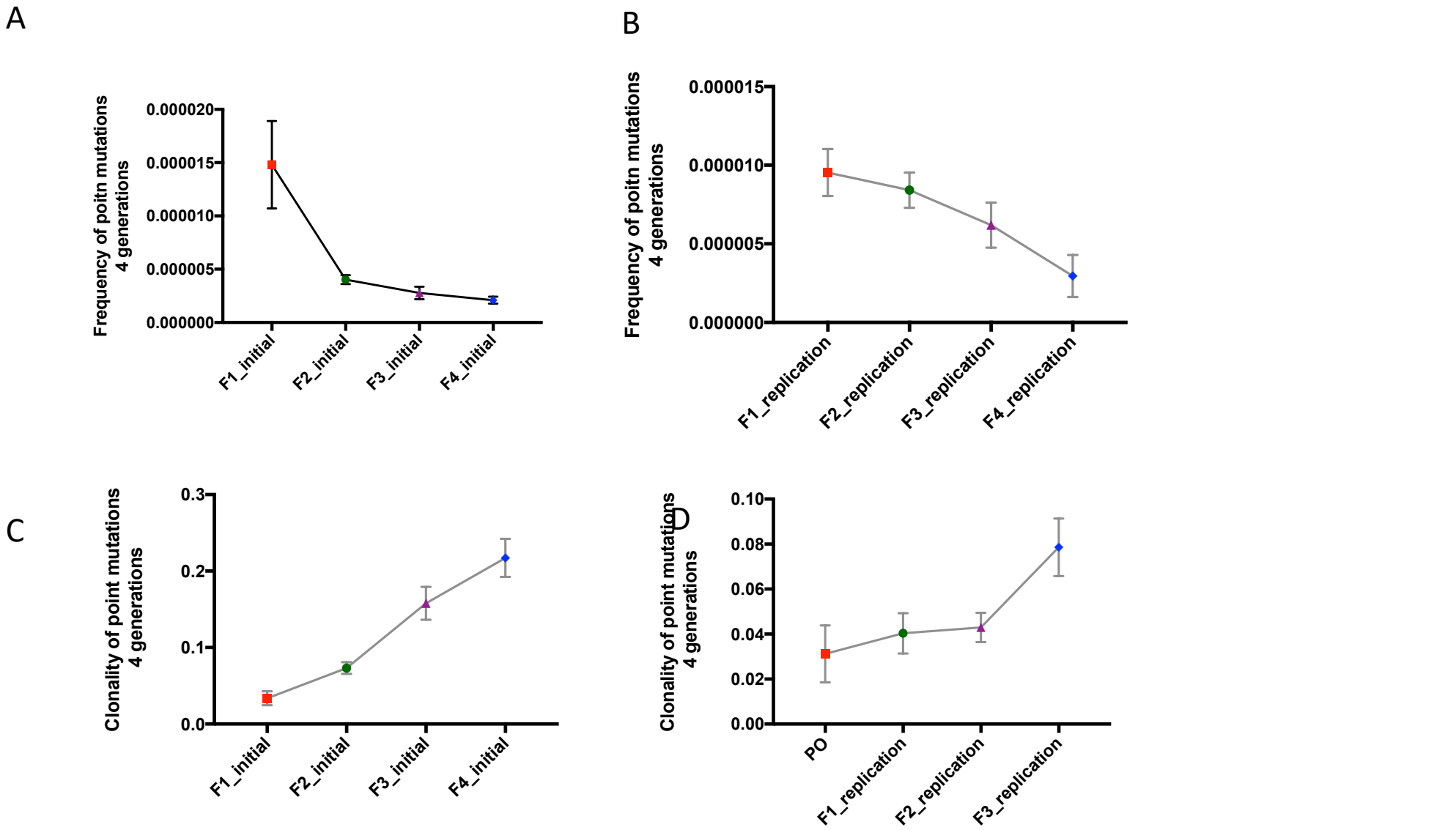


Figure 7: (A) and (B)The frequency of mtDNA point mutations drops gradually with each generation in two independent lineages; (C) and (D) whereas the clonality of these mutations increases in the same independent lineages.

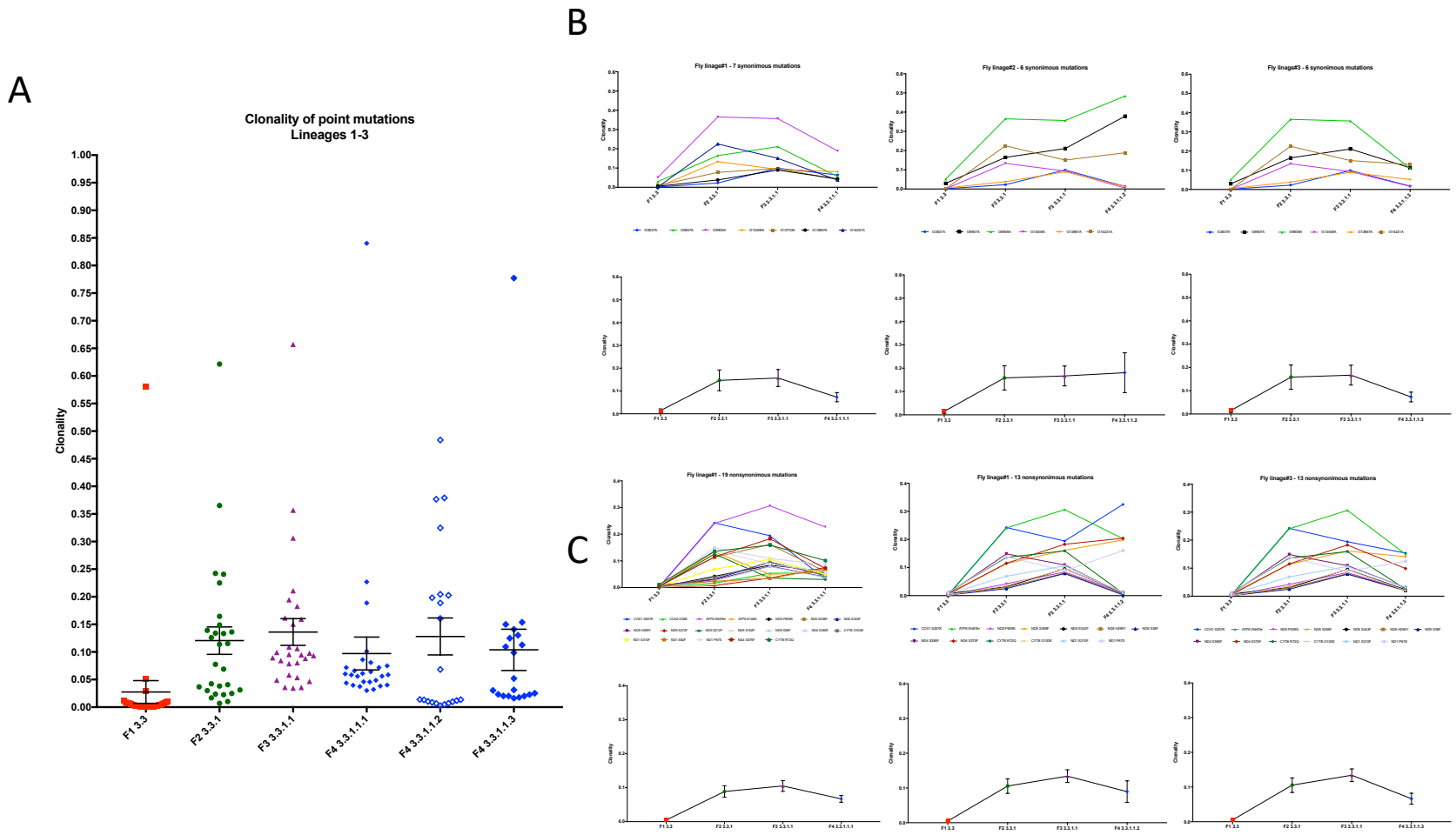


Figure 8: Following of specific mutations that are transmitted through the germline in multiple generations starting in the F1. (A) Clonality of all shared mutations as a function of generation. (B) *top row:* clonality changes of synonymous mutations across 4 generations. *Bottom row:* average values. (C) *top row:* clonality changes of non-synonymous mutations across 4 generations.

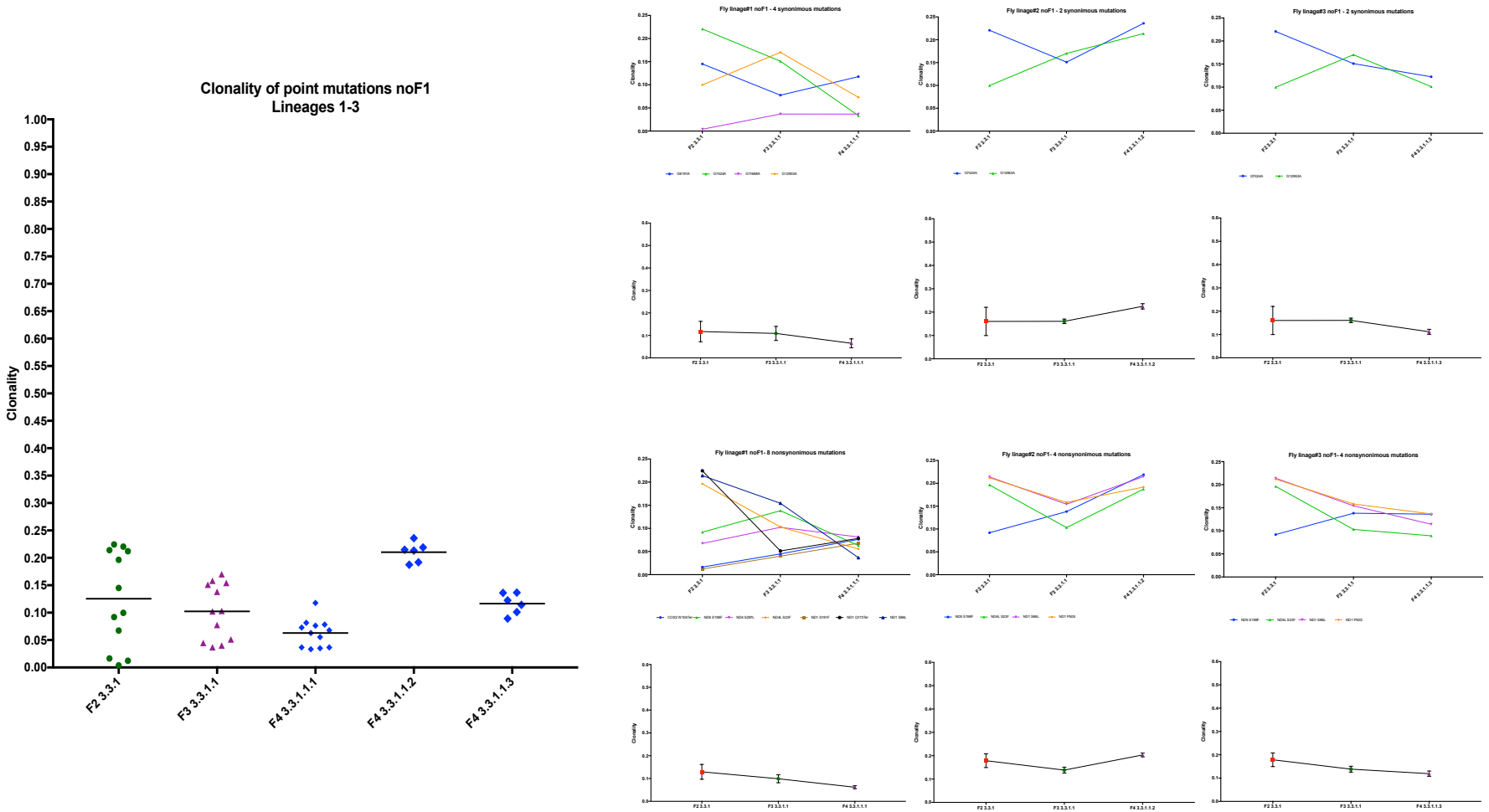


Figure 9: Following of specific mutations that are transmitted through the germline in multiple generations starting in F2. (A) Clonality of all shared mutations as a function of generation. (B) *top row:* clonality changes of synonymous mutations across 4 generations. *Bottom row:* average values. (C) *top row:* clonality changes of non-synonymous mutations across 4 generations.

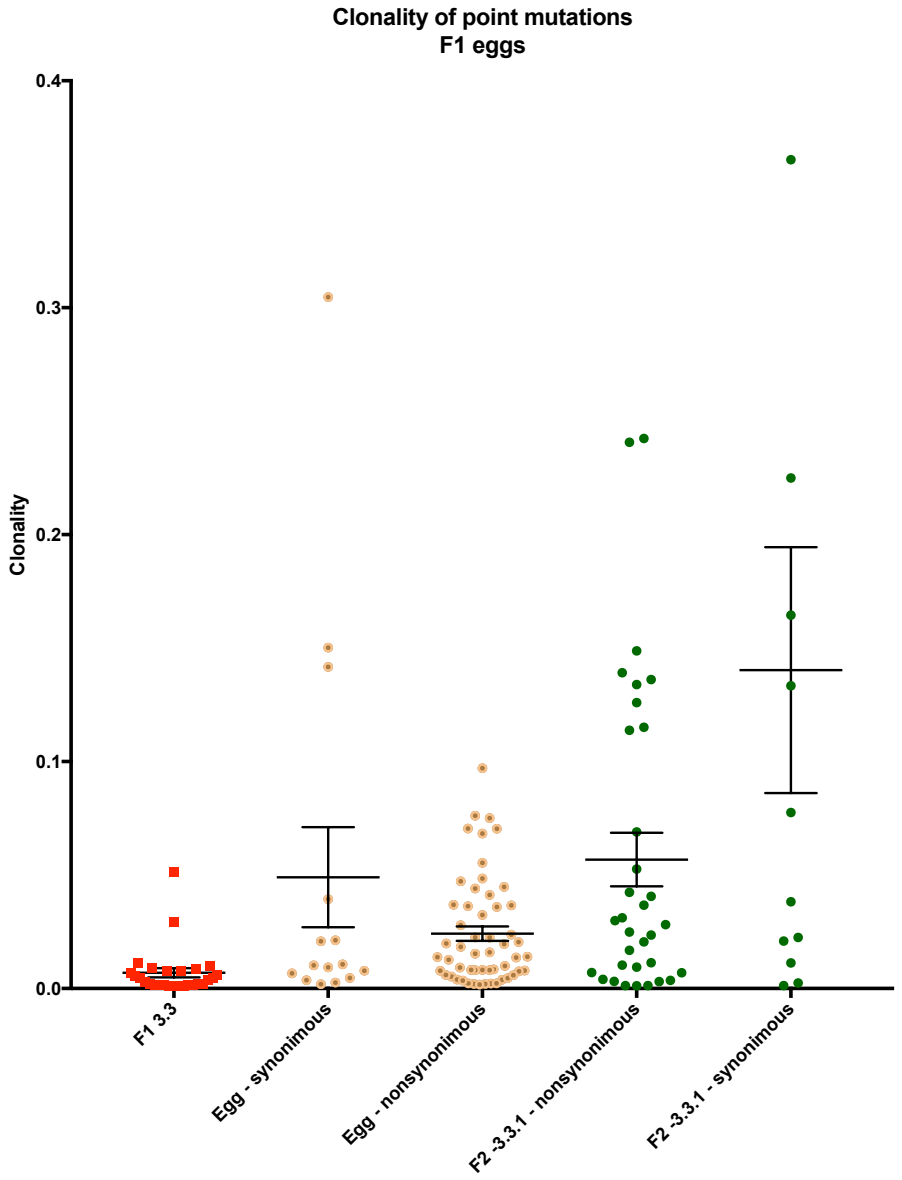


Figure 10: Clonality of synonymous and nonsynonymous mutations in one single egg from an F1 and resulting F2 offspring from the same mother as the eggs.

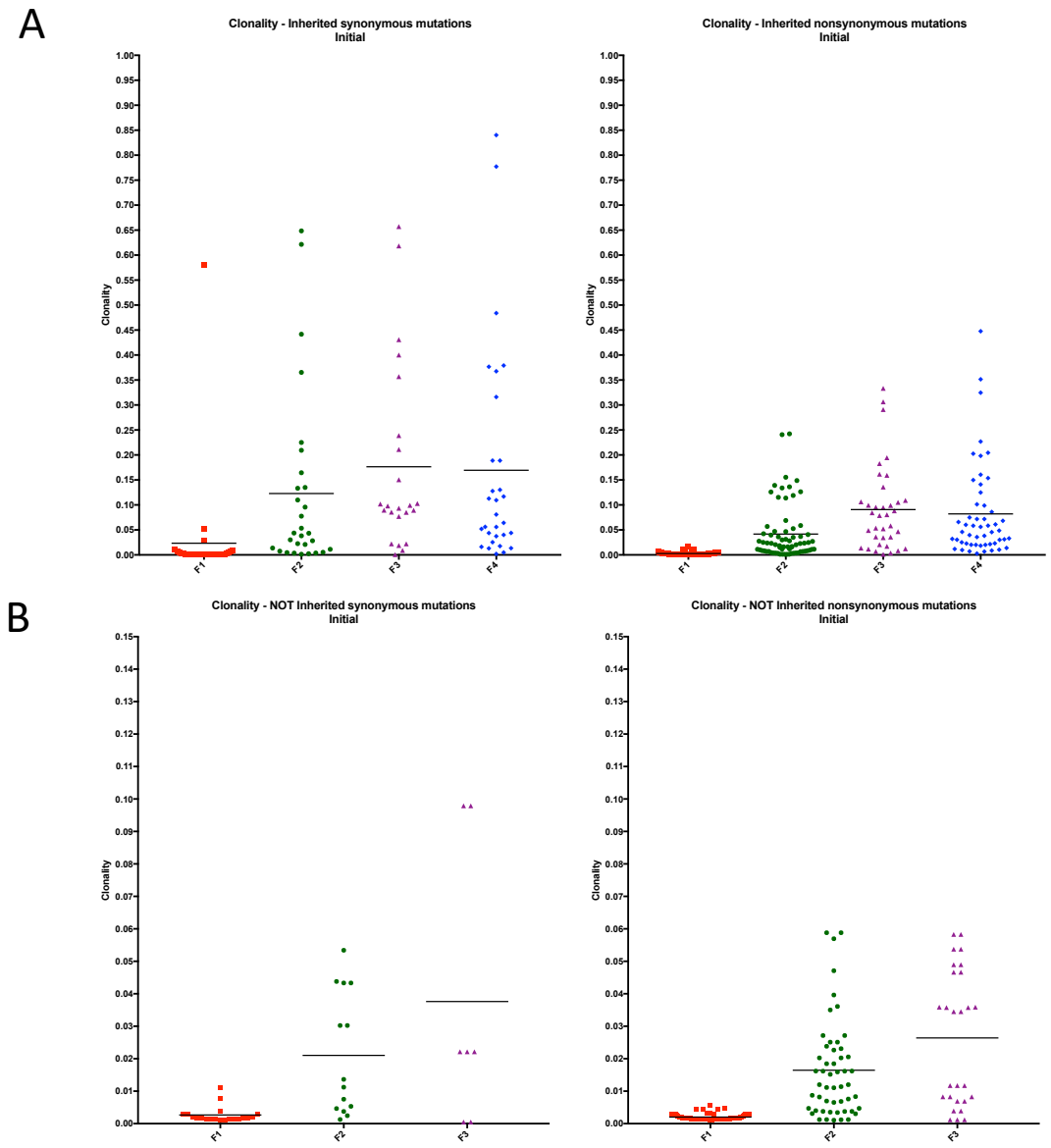


Figure 11: Clonality levels of synonymous and non-synonymous mutations. (A) Clonality of inherited mutations. (B) Clonality of non-shared mutations.

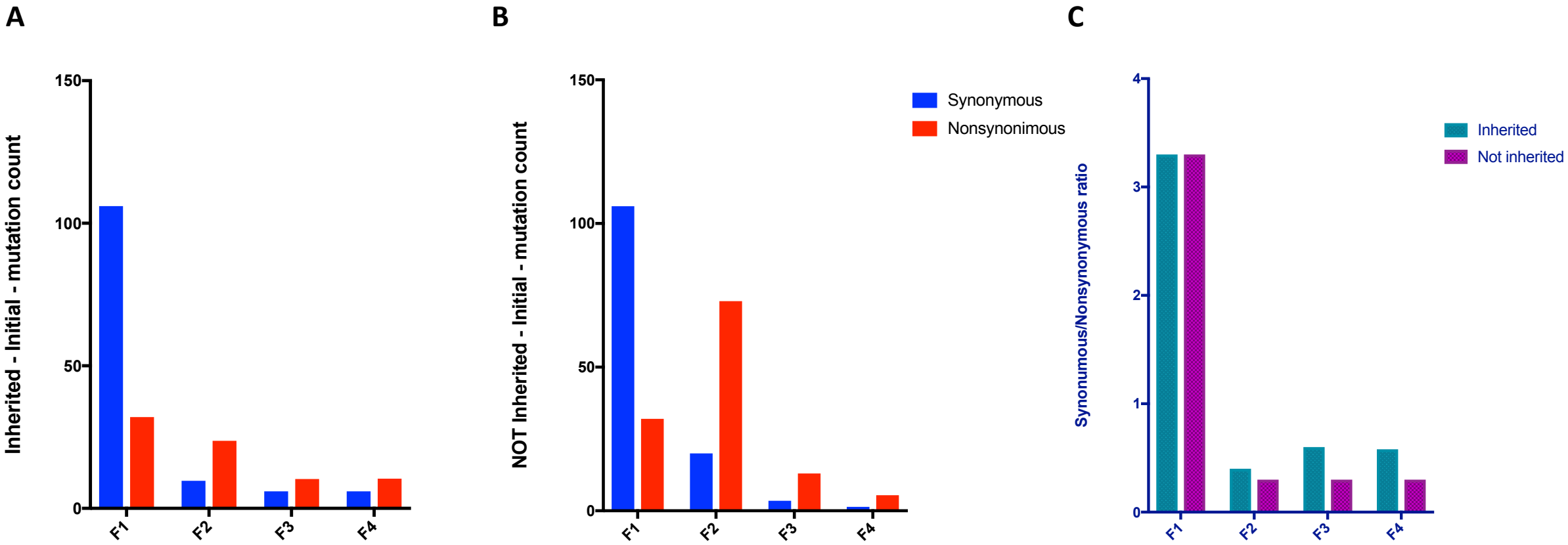


Figure 12: Synonymous/Non-synonymous ratios across four generations. (A) Mutation counts of synonymous and non-synonymous mutations that are inherited. (B)) Mutation counts of synonymous and non-synonymous mutations that are non-inherited. (C) Synonymous to non-synonymous ratio of inherited and non-inherited mutations.

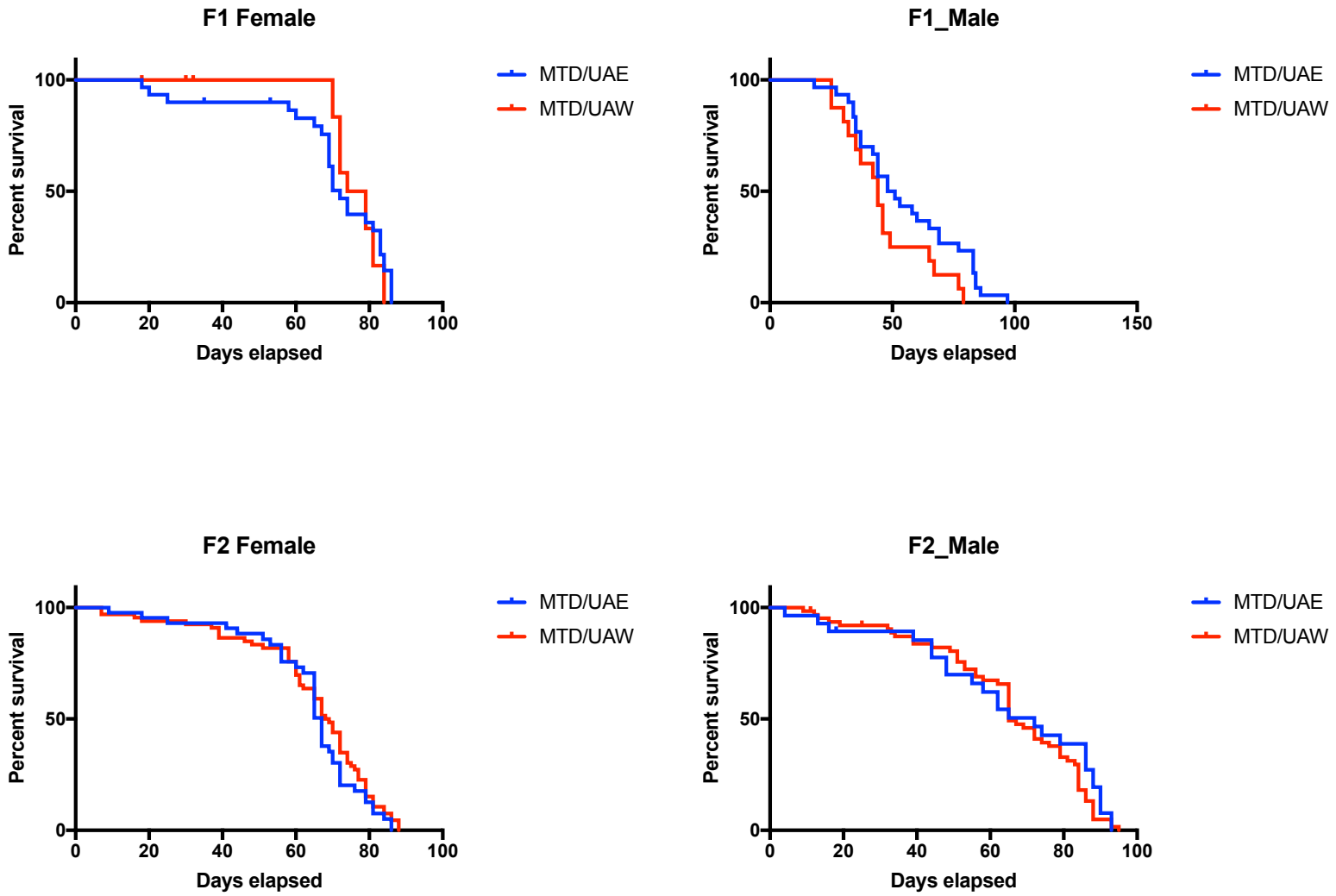


Figure 13: Lifespan analysis of of APOBEC1 (*red*) and catalytically dead APOBEC1 (*blue*).