Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA

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Mitochondrial DNA (mtDNA) is maternally inherited in mammals. Despite the high genome copy number in mature oocytes (10^5) and the relatively small number of cell divisions in the female germline, mtDNA sequence variants segregate rapidly between generations. To investigate the molecular basis for this apparent paradox we created lines of heteroplasmic mice carrying two mtDNA genotypes. We show that the pattern of segregation can be explained by random genetic drift ocurring in early oogenesis, and that the effective number of segregating units for mtDNA is ~200 in mice. These results provide the basis for estimating recurrence risks for mitochondrial disease due to pathogenic mtDNA mutations and for predicting the rate of fixation of neutral mtDNA mutations in maternal lineages.

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Mammalian mtDNA is a 16.5 kb circular molecule which contains genes encoding 13 polypeptides involved in oxidative phosphorylation, and a minimal compliment of 22 tRNAs and 2 rRNAs used by the mitochondrial translation machinery. All other components for the function and maintainance of mitochondria are encoded by nuclear genes. The rate of nucleotide substitution in the mammalian mitochondrial genome is five to ten times faster than in the nuclear genome[1,2]. As a consequence, mtDNA is highly polymorphic. However, most genetic variance within a species exists between individuals and the occurrence of more than one mtDNA sequence variant in an individual (heteroplasmy) is rare. Pedigree analysis of a heteroplasmic D-loop sequence variant in Holstein cows has demonstrated a return to homoplasmy within two generations[3–6], and in some maternal lineages, complete allele switching has been observed in a single generation, without a heteroplasmic intermediate[7]. Analysis of human pedigrees segregating pathogenic mtDNA point mutations also shows large differences in the proportion of mutant mtDNAs among siblings and between generations[8–10].

To account for the rapid segregation of mtDNA sequence variants a genetic bottleneck for mtDNA in the female germline or early embryo has been proposed. Two molecular mechanisms have been hypothesized: 1) During maturation of the primary oocyte the number of mtDNA molecules increases 100-fold from approximately 10^3 to 10^5 (ref. 11 and unpublished results). Selection and subsequent replication of a small subpopulation of DNA templates during this process could produce rapid shifts in mtDNA genotype frequencies in a single generation[3]. 2) Preimplantation embryos undergo several cell divisions prior to initiating mtDNA replication[11]. At the early blastocyst stage a small number of cells (10–20) (ref. 12), each containing ~10^3 mtDNAs, contribute to the inner cell mass that gives rise to all three germ layers of the embryo, while the other cells in the blastocyst give rise to extraembryonic tissues. Unequal partitioning of mtDNAs to the inner mass cells at this stage of embryogenesis could also contribute to rapid segregation of mtDNA genotypes[4,5].

We tested these hypotheses using lines of heteroplasmic mice carrying two mtDNA genotypes and show that neither are correct in these mice. Rather, we show that segregation of mtDNA occurs by random genetic drift in early oogenesis in the small population of mtDNAs present in oogonia (the precursors of primary oocytes), and that the effective number of segregating units is approximately 200. Analysis of several human pedigrees segregating pathogenic mtDNA point mutations suggest that the process is substantially similar in humans.

Segregation of mtDNA in heteroplasmic mice

We produced embryos carrying two mtDNA genotypes by electrofusing cytoplasts recovered from NZB/BinJ (NZB) or BALB/cByJ (BALB) derived zygotes to recipient one-cell embryos of the other type. They were maintained in culture and transplanted as two-cell embryos into pseudopregnant B6C3F1 females to complete development. We identified five female and nine male animals carrying mtDNA of both genotypes by analysis of mtDNA from tail biopsies. All five founder females, carrying from 3.1–7.1% of the donor mtDNA genotype, were mated to BALB males and the resulting

Table 1 Segregation of mtDNA in the offspring of founder and F1 females

Female	Donor mtDNA genotype	p_o(×10²)	n	p_n(×10²)	range	CV	N
		F1 offspring					
455	BALB	3.1	35	4.8	0–24.6	1.54	76
511	NZB	5.0	28	6.7	0–29.6	1.08	126
515	BALB	3.2	48	5.1	0–18.6	0.96	185
517	BALB	6.3	43	7.6	0–22.0	0.88	193
506	NZB	7.1	37	7.4	0–26.0	0.63	449
		BC1 offspring					
531	BALB	17.2	18	29.1	14.6–51.6	0.39	155
539	NZB	7.6	34	4.7	0–17.4	0.98	480
527	BALB	7.1	20	8.2	2.1–18.1	0.60	394
530	BALB	3.1	48	5.8	0–24.8	1.19	87
611	NZB	4.4	31	6.9	0–26.5	1.00	125
603	NZB	5.6	25	2.0	0–11.1	1.49	867

P_o and P_n, frequency of the donor mtDNA genotype in the mother and the mean frequency in her offspring. n, number of pups. Range, range in the frequency of the donor mtDNA genotype (×10²) present in individual offspring. CV, coefficient of variation in the frequency of mtDNA genotypes. N, the effective number of segregating units for mtDNA.

F1 progeny were genotyped. The proportion of donor mtDNA genotype in a given female was similar to the mean proportion in all her offspring (Table 1). However, in individual offspring substantial shifts in the proportion of the two mtDNA genotypes were observed (Table 1, Fig. 1*a*). This was true when either NZB or BALB was used as the donor mtDNA genotype. The donor genotype was undetectable in some offspring from all founder females (fixation of the recipient mtDNA genotype); other offspring contained as much as 29.6% of the donor genotype (Table 1). This is reflected in the large variance of genotype frequencies observed amongst the progeny derived from individual females (Table 1). To allow comparison of the offspring from females carrying different mean frequencies of the donor genotype these data are shown as the coefficient of variation. Similar results were observed in the progeny from six females in the first backcross generation (BC1, F1 females × BALB males) (Table 1). These data demonstrate that germline segregation of the mtDNA genotypes used here is a stochastic process and establish the suitability of the marker for studies of mitochondrial inheritance.

Molecular basis for rapid segregation of mtDNA

To determine the molecular basis for the rapid segregation of mtDNA genotypes in the offspring of heteroplasmic females we used single-cell PCR techniques to measure the proportions of the two mtDNA genotypes at different stages of oogenesis. We compared the variance in the frequency of mtDNA genotypes at each stage of oogenesis to that in somatic tissues in the offspring.

To test whether segregation of mtDNA occurred at any stage of post-fertilization development we compared the distribution of mtDNA genotypes in mature oocytes and F1 offspring from the same founder females. We observed no significant difference in the range of donor mtDNA proportions or in the coefficient of variation in the proportion of mtDNA genotypes in these two populations (Fig 1*a*, Table 2). Thus, unequal partitioning of mtDNAs into the inner cell mass during early cleavage of the zygote[4,5] does not contribute significantly to segregation of mtDNA genotypes between generations. The relative proportions of the two mtDNA genotypes in an individual must therefore be determined at an earlier stage of development.

If only a small number of templates were used during the 100-fold mtDNA amplification that occurs during oocyte maturation one would expect to observe a large increase in genetic variance in the population of mature oocytes compared to that in immature oocytes. To test this hypothesis we first compared the variance in the frequency of mtDNA genotypes in primary oocytes from immature (postnatal day 2–3) heteroplasmic mice to that in mature

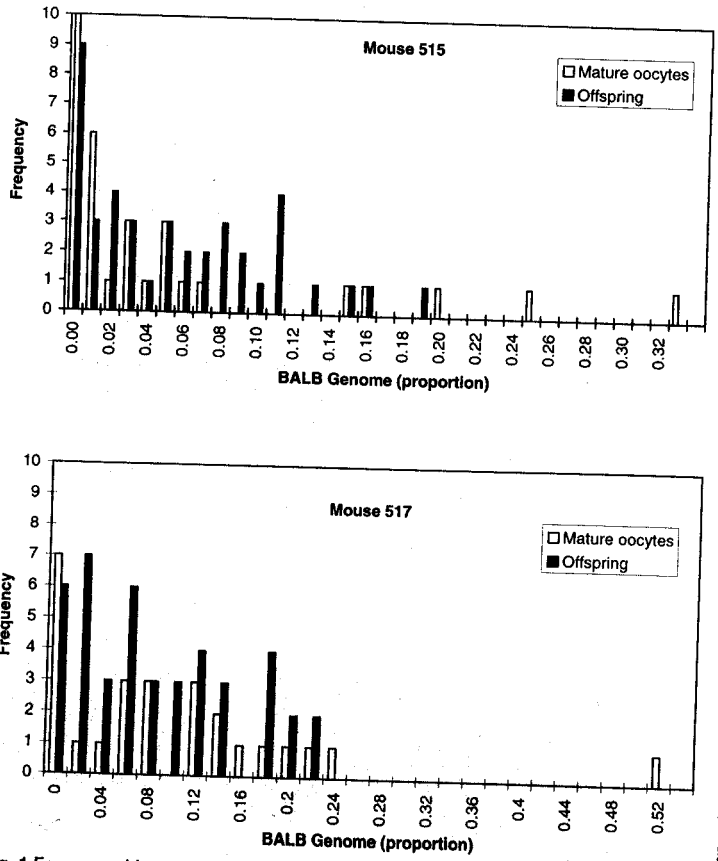

Fig. 1 Frequency histogram of the distribution of mtDNA genotypes in individual F1 offspring and mature oocytes from two founder females.

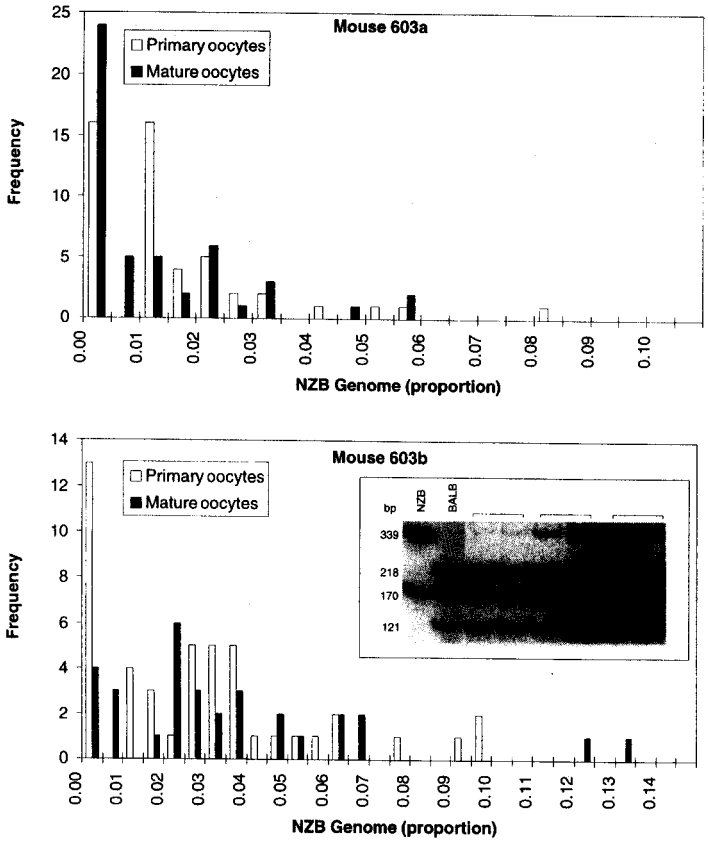

Fig. 2 Frequency histograms of the distribution of mtDNA genotypes in primary and mature oocytes from the same animals. Results of two experiments are shown. The inset in the bottom panel shows control data from DNA extracted from tail biopsies of homoplasmic animals and three duplicate samples from primary oocytes.

mtDNA was developed from segregation analysis of mtDNA sequence polymorphisms in Holstein cows[3–5]. However, estimates of *N*, based on the segregation of a D-loop sequence variant, are at least five times lower (at equivalent *g*) than what we calculate for the mouse. Further, in one such study many mother offspring pairs were shown to be homoplasmic for alternate mtDNA genotypes, implying complete segregation in a single generation without a heteroplasmic intermediate, and leading to the suggestion that the size of the segregating unit may be as small as a single mtDNA molecule[7]. The D-loop sequence variant examined in all of the cow studies is adjacent to a homopolymeric tract of G's and because the length of the tract of G's is variable, the polymorphism could be interpreted as a G–C transversion or a C insertion. The discrepancy between results obtained in the cow and mouse could be due to a species difference in the mechanism of mtDNA transmission or, alternatively, it could reflect a high mutation rate or genetic instability in this region of the D-loop. In support of the latter notion, heteroplasmy, which is generally rare, has been observed at this site in a large number of independent maternal Holstein lineages[4,6,7] and somatic cell mosaicism for length variants has been reported in the tract of Gs[24]. Although heteroplasmy has not been observed directly for any other sequence variant in mtDNA in cows, pedigree analysis indicates that fixation of a polymorphism in a coding sequence in a maternal lineage requires three or more generations[3].

MtDNA heteroplasmy in humans has been found in association with a heterogeneous group of disorders referred to as mitochondrial encephalomyopathies[25]. Analysis of human pedigrees segregating pathogenic point mutations in mtDNA coding sequences demonstrates that complete segregation of mild mutations (homoplasmy), and large shifts in the proportion of mutant mtDNAs associated with severe phenotypes (always heteroplasmic), can occur in some individuals in as little as 3–4 generations[8–10]. Although homoplasmy for a severe pathogenic mtDNA mutation has never been observed in any individual, presumably because the condition is lethal, the proportion of mutant mtDNAs in a heteroplasmic individual must generally exceed 85–90% before a biochemical or clinical phenotype is expressed[26,27]. Below this threshold mutant mtDNAs should be invisible to selection and would likely segregate randomly. This hypothesis can be tested by calculating *N*, as above, from completely genotyped pedigrees with few affected individuals. In humans an estimated 6 million primary oocytes are produced[28] which would require ~24 mitotic divisions from a single founder cell. Data from human pedigrees must, however, be evaluated with caution for several reasons. First, the pedigree size is usually small and therefore the estimate of the variance in the frequency of different genotypes is poor. Second, because of an ascertainment bias, the data is skewed to include pedigrees with clinically affected individuals which will tend to exaggerate the apparent rate of segregation. The bias will also tend to increase the frequency of the mutant mtDNA in later generations and, when this is severe, to preclude calculation of *N* using the random drift model for obvious reasons. Finally, the mtDNA genotype is often only available from analysis of blood, which may not be representative of the overall level of heteroplasmy in the individual. With these constraints in mind we have estimated *N* from several published pedigrees. In individuals with the A8344G $tRNA^{lys}$ mutation associated with the myoclonus epilepsy and ragged-red fibre syndrome (MERRF) the proportion of mutant mtDNAs in blood correlates well with that in other tissues[29]. Using the data from two branches of a very large MERRF pedigree we calculate *N*'s of 140, 349, and, from another independent pedigree, 135 (ref. 8). The relationship between the level of heteroplasmy in blood and other tissues is less clear in families segregating mutations associated with Lebers hereditary optic neuropathy (LHON) or the neuropathy, ataxia, retinitis pigmentosa syndrome (NARP). Nonetheless, using data from three LHON (G11778A) pedigrees we calculate *N* to be 164 (ref. 30), 110 (ref. 31) and 316 (ref. 32), from one NARP (T8993G) pedigree to be 24 (ref. 10) and from one small family with a silent polymorphism

Table 2 Segregation of mtDNA in the female germ line

Mother	Donor mtDNA genotype	p_o(×10²)	n	p_n(×10²)	range	CV
		Mature oocytes				
517	BALB	6.3	26	9.4	0–50.5	1.16
541	BALB	10.0	24	10.0	0–17.9	0.39
515	BALB	3.2	31	4.7	0–32.4	1.71
603A[a]	NZB	1.4	49	0.9	0–5.2	1.51
603B[a]	NZB	4.0	31	3.1	0–12.9	1.01
		Primary oocytes				
527A	BALB	7.3	40	10.1	1.4–25.5	0.59
531A	BALB	17.2	37	22.7	13.1–46.7	0.33
531B	BALB	38.9	35	37.0	17.1–56.9	0.26
783a1	BALB	3.8	35	4.6	0–16.8	0.89
783a3	BALB	4.2	34	5.3	0–13.8	0.81
603A	NZB	1.4	48	1.1	0–7.8	1.38
603B	NZB	4.0	45	2.5	0–9.3	1.01
		Primordial germ cells				
819c	NZB	4.6	12	4.9	2.2–8.3	0.01
791a1	BALB	38.6	4	40.3	33.4–45.0	0.11
791a3	BALB	26.6	3	30.9	27.6–32.7	0.09
791a5	BALB	34.9	5	39.3	31.7–41.8	0.11

Symbols are the same as in Table 1, except that n refers to the number of individual cells.
[a]Mature oocytes recovered from the transplanted ovary.

oocytes from adult mice. The coefficient of variation was similar in these two populations (Table 2). Second, in order to directly compare mtDNA genotypes in primary and mature oocytes from the same heteroplasmic animal, we obtained immature oocytes from one ovary on postnatal day 2 and mature oocytes from the other ovary after it was transplanted and allowed to mature in the ovarian capsule of a nude mouse. The coefficient of variation was virtually identical in primary and mature oocytes derived from the same animal (Table 2). These results demonstrate that the variance in the primary oocyte population is sufficient to explain the variance in mtDNA genotype frequencies in adults of the next generation. Segregation of mtDNAs must therefore occur even earlier in oogenesis, prior to differentiation of the primary oocyte population.

Before the the primary oocyte population is established the germ cell lineage consists of approximately 50 primordial germ cells (PGCs) located at the base of the allantois in postcoitum 7.5 day mouse embryos. These cells can be identified using histochemical techniques by virtue of their high alkaline phosphatase activity[13]. The PGCs replicate and migrate to the genital ridge where in females they give rise to the population of oogonia. The oogonia undergo a series of synchronous mitotic divisions differentiating into approximately 25,000 primary oocytes[14]. In sharp contrast to results obtained in primary and mature oocytes, our analysis of the mtDNA genotype in PGCs showed that the coefficient of variation in these cells was very small (Table 2). Thus, segregation of mtDNA sequence variants must occur during expansion of the oogonial population.

Discussion

The results of this study demonstrate, contrary to current hypotheses, that the rapid segregation of mtDNA occurs during early oogenesis and is essentially complete by the time the primary oocyte population has differentiated. It is most likely that segregation occurs during the rounds of synchronous mitotic divisions that occur in the population of oogonia as they differentiate into primary oocytes. Two mechanisms contribute to mitotic segregation of mtDNA: relaxed control of mtDNA replication permitting a single mtDNA molecule to replicate more than once or not at all during a single cell cycle, and random partitioning of mitochondria to daughter cells during cytokinesis[15,16]. Thus the rate of segregation of mtDNA is a function of the mtDNA copy number in individual cells, the number of mtDNAs that are used as templates for replication and the number of mitotic divisions in the lineage. Human and other mammalian oogonia have a sparse population of mitochondria[17]. For the mouse we estimate that oogonia contain only about 40 mitochondria. This number was calculated from data in Nagawa *et al.*[18] using the numbers of mitochondria determined on cross-sections of oogonia, assuming the cell to be a sphere and the mitochondria uniformly distributed. While the precise number of mtDNAs/organelle in oogonia is not known, in somatic cells it averages five copies per organelle[19]. Based on these estimates oogonia would have roughly 200 mtDNAs, which is low relative to that determined in several other cell types[20,21].

The segregation of mitochondrial and chloroplast genomes in cells and organisms has been modelled previously using population genetic theory[22,23]. By measuring the change in variance of mtDNA genotype frequencies between generations one can estimate the effective number mtDNAs that are sampled at each generation from a large pool, assuming that segregation occurs by genetic drift. We calculated the effective number of segregating units (N) for mtDNA using such a model which relates the variance (V_n) in the nth generation to the initial frequency of one of the mtDNA genotypes (p_o) taking account of the number of cell divisions in the female germline (g) as follows:

$$V_n = p_o(1-p_o)\left[1-\left(1-\frac{1}{N}\right)^{gn}\right]$$

About 15 cell divisions would be necessary to produce the population of primary oocytes in the mouse even if we consider this lineage to arise from a single founder cell. Using g=15 and the data on genotype frequencies and variance in Table 1, the median effective number of segregating units (N) for mtDNA in mice, calculated from the five F1 and six BC1 litters, is 185 (range, 76–867) (Table 1) which is close to the estimated mtDNA copy number in oogonia[18,19]. As N is only a statistical estimate of effective sample size, this close correspondence may be fortuitous and does not imply that mtDNAs in oogonia segregate as independent units. Nonetheless, our analysis strongly suggests that the rapid stochastic segregation of mtDNA genotypes is primarily due the relatively small number of mtDNA templates in oogonia, before they mature into primary oocytes.

The concept of a genetic bottleneck for mammalian

(G14560A) to be 89 (ref. 33). In spite of the potential biases in the data, these estimates are within or near the range predicted from the mouse model, making it likely that segregation of germline-transmitted pathogenic mtDNA mutations in humans is also largely due to random genetic drift.

Our study suggests that the probability of inheriting one of two mtDNA genotypes can be modelled as a binomial sampling process. Based on the analysis of human pedigrees, it seems unlikely that strong positive or negative selection for pathogenic mtDNAs occurs in the oocyte or in early embryogenesis below the threshold for expression of a biochemical phenotype. It should thus be possible to predict recurrence risks of mitochondrial disease from the proportion of mutant mtDNA in a female carrier. Further, it should be possible to provide preimplantation diagnosis based on the mtDNA genotype determined from a single blastomere.

The random drift model can also be used to predict the rate of fixation or loss of new mtDNA mutations in a maternal lineage. Using 185 as an estimate for N in humans the mean time to fixation of a new mutation in a maternal lineage is approximately 370 cell generations or about 15 sexual generations[22]. The rate of fixation of a neutral mtDNA allele in a population is a function of the number of females. It is thought that the effective human population size has not been lower than 5,000 females in the past 1Myr[34], which predicts fixation of a neutral allele in approximately 10,000 generations. The mean rate of return to homoplasmy for any new allele is thus very rapid compared to the rate of fixation of the mutation in human populations, explaining why polymorphism is common, but heteroplasmy is rare.

Methods

Generation of heteroplasmic animals. Embryos carrying two mtDNA genotypes were produced using methods analagous to those developed for pronuclear transfer[35]. The zona pellucidae were removed from one-cell embryos with 0.5% pronase for 5 min at 37 °C. The embryos were washed twice with Brinster's BMOC medium, then incubated in 5 μg/ml cytochalasin B and 0.1 μg/ml actinomycin D for 30 min. Cytoplasts were produced by drawing up and pinching off cytoplasm surrounded by the plasma membrane in a 15–20 μm glass pipette. Donor cytoplasts were injected under the zona pellucida of recipient one-cell embryos which had been treated with 5 μg/ml cytochalasin B and 0.1 μg/ml colcemid for 30 min. To form the cybrid (cytoplasmic hybrid) embryos cytoplasts were electrofused to zygotes with 2 x 50 ms square wave pulses at 2–4 kV/cm in BMOC medium in a chamber with a 0.2 mm gap. The embryos were cultured overnight at 37 °C, 5% CO_2 in BMOC medium and transplanted as two cell embryos into the fallopian tubes of B6C3F1 females on day 1 of pseudopregnancy, induced by mating with a vasectomized males. Because NZB mice have poor reproductive performance and develop autoimmune disease we generated NZB/BALB F1 mice carrying the NZB mtDNA genotype (female NZB x male Balb). These F1 mice were backcrossed to BALB males and zygotes from animals in the BC1 to BC3 generations were used in all studies. Thus the average NZB nuclear background would vary from 6.25–25% in mice with the NZB mtDNA genotype.

mtDNA genotype analysis. An *RsaI* site at position 3961 in the *ND2* gene present in BALB but absent in NZB mice was used to genotype individual animals[36]. The mtDNA genotype was determined on a PCR-amplified fragment encompassing the polymorphic site. Genomic DNA was amplified using standard conditions and the following primers: forward, nt 3571–3591, 5'–GAGCATCTTATCCACGCTTCC–3'; reverse, nt 4079–4059, 5'–CTGCTTCAGTTGATCGTGGGT–3' as follows: 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s for 30 cycles. In the last cycle 1.5 μCi of [α^{32}]P-dCTP was added to the reaction to radiolabel the PCR product. A 15 μl sample of this reaction was cut with 10 U *RsaI* overnight and run on a 10% nondenaturing polyacrylamide gel (Inset Fig. 2). Gels were analysed on a Fuji 1000 phosphorimager. The proportion of the donor mtDNA genotype in founder and F1 mothers was determined from the mean of at least five non-mitotic tissues and was not significantly different from that determined on tail biopsy specimens. The mtDNA genotype was determined from tail biopsies in all offspring.

Oocyte and PGC isolation. Mature and primary oocytes were isolated as described[37]. Primary oocytes were collected from post-natal day 2–4 animals and mature oocytes from ovaries in mature animals. Oocytes were extracted for PCR analysis as decribed[38]. Of the final volume, 40% was used to amplify the mtDNA from mature and primary oocytes. A nested PCR approach was used to amplify the mitochondrial DNA from the primary oocytes. The following primers were used for the first round: forward, nt 3509–3529, 5'–TCTACTCAACTAACTTCATAA–3'; reverse, nt 4194–4174, 5'–GGATAAGACCGTTTGTTTGTT–3', using the conditions described earlier and cycled for 15 cycles as follows: 94 °C, 30 s; 48 °C, 30 s; 72 °C 1 min. A 0.5 μl aliquot was used for the second round amplification using primers and conditions described earlier. To ensure that genotype frequencies in the PCR-amplified product reflected those in the population of DNA templates, DNA from mature oocytes was extracted from each founder strain, mixed and PCR carried out on serial dilutions. Duplicate samples from serial dilutions were analysed and significant differences between duplicates were not seen until less than ~100 mtDNA copies were amplified. Primary oocytes from several animals (603A, 603B and 527A) were analysed in duplicate to control for accidental loss of template DNA during transfer and extraction, and an average of the two determinations was used in the analysis (Inset Fig. 2). Data were included in the analysis if duplicate values were within ±10% of the mean using an estimated absolute measurment error of ±2%; 94% of primary oocytes were within this range.

Primordial germ cells (PGC's) were isolated from embryonic day 8.5 animals. Tissue dissected from the posterior third of the embryo was dispersed into a cell suspension while staining in 1 ml of 0.5 M tris-maleate pH 9.0, 0.4 mg/ml α-napthyl phosphate, 1 mg/ml Fast-Red TR salt for 5–10 min[13]. The reaction was stopped with 1 ml of 100% ethanol. The PGC's were retrieved by virtue of dark staining due to high alkaline phosphatase activity. Single PGC's were extracted as above and the mtDNA genotype was determined in duplicate samples using nested PCR.

Ovarian transplant. Both ovaries from P2-3 females were removed and one was transplanted into the ovarian capsule of an oophorectomized transplant tolerant nude (*nu/nu*) mouse. The transplanted ovary was recovered after 1 month and oocytes greater than 70 μm were isolated. Primary oocytes were collected from the non-transplanted ovary.

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