Somatic instability of the expanded GAA triplet-repeat sequence in Friedreich ataxia progresses throughout life

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ABSTRACT

Friedreich ataxia (FRDA) patients are homozygous for expanded GAA triplet-repeat alleles in the *FXN* gene. Primary neurodegeneration involving the dorsal root ganglia (DRG) results in progressive ataxia. While it is known that DRG are inherently sensitive to frataxin deficiency, recent observations also indicate that they show age-dependent, further expansion of the GAA triplet-repeat mutation. Whether somatic instability is progressive has not been systematically investigated in FRDA patients. Small pool PCR analysis of ~2300 individual molecules from tissues of an 18-week fetus, homozygous for expanded alleles, revealed very low levels of instability compared with adult-derived tissues (4.2% versus 30.6%, $P<0.0001$). Mutation load in blood samples from multiple patients and carriers increased significantly with age, ranging from 7.5% at 18-weeks gestation to 78.7% at 49y ($R=0.91$; $P=0.0001$). Therefore, somatic instability in FRDA occurs mostly after early embryonic development and progresses throughout life, lending further support to the role of postnatal somatic instability in disease pathogenesis.

Keywords: Friedreich ataxia; GAA triplet-repeat; instability; progressive; embryonic development; somatic instability.

INTRODUCTION

Friedreich ataxia (FRDA) is characterized by slowly progressive sensory ataxia with onset usually before the age of 25y, typically associated with absent tendon reflexes, loss of position and vibration senses, dysarthria, and extensor plantar responses [1, 2]. These neurological manifestations result from primary degeneration of the dorsal root ganglia (DRG), associated with axonal degeneration in the posterior columns, spinocerebellar tracts, and the corticospinal tracts of the spinal cord, and large myelinated fibers in the peripheral nerves [3]. Although the rate of progression is variable, the mean age of loss of ambulation is 25y, and patients often die in their fourth or fifth decade.

FRDA is inherited as an autosomal recessive trait and the vast majority of patients are homozygous for expanded GAA triplet-repeat (GAA-TR) sequences (E alleles) in intron 1 of the *FXN* gene [4]. E alleles interfere with *FXN* gene transcription in a length-dependent manner [5, 6] and result in proportional deficiency of frataxin [7]. Most E alleles contain 600 – 1200 triplets, however, the spectrum of disease-causing alleles ranges from 66 to 1700 triplets. Disease severity and rate of progression correlate with the length of E alleles [8, 9, 10, 11, 12], and carriers of shorter alleles (<500 triplets) commonly show slower than average disease progression.

DRG neurons are especially sensitive to frataxin deficiency as was demonstrated by their selective loss in neuronal-specific, conditional, frataxin knockout mice [13]. Upon analysis of nervous system tissue from multiple autopsies of FRDA patients we recently found that there was a progressive accumulation of large expansions in the DRG [14]. This phenomenon was not seen in other tissues and may serve as a further

modulator of the progressive DRG-specific pathology seen in FRDA patients. Furthermore, in patients who are compound heterozygous for a conventional E allele and a borderline GAA-TR allele (44 – 66 triplets), somatic instability of the borderline allele was required for the development of the FRDA phenotype [15].

All of the above observations suggest that somatic instability of E alleles in FRDA is likely to be progressive. However, this has not been systematically investigated in patient-derived tissues. Given that DNA replication is able to induce instability of GAA-TR sequences in simple model systems [16, 17], one would predict that the burst of cellular proliferation occurring in early embryonic development would be associated with enhanced somatic instability. Here we show that somatic instability of E alleles is progressive; it occurs mostly after early embryonic development and continues throughout life. These observations have important implications for the mechanism of somatic instability in FRDA, and lend further support to the role of progressive somatic instability in disease pathogenesis.

RESULTS

Somatic instability in FRDA mainly occurs after early embryonic development

E alleles are commonly detected by conventional PCR or genomic Southern blot analysis, which typically use 0.06 – 6 μg genomic DNA, thus simultaneously analyzing 10^4 – 10^6 cells. These assays, commonly used for molecular diagnosis of patients, only estimate the repeat length of the "constitutional" or "most common" allele (E allele). However, using small pool PCR (SP-PCR), a sensitive assay that involves the use of very low levels of genomic DNA (typically 6 – 600 pg), it is possible to detect GAA triplet-repeats in individual *FXN* molecules (genes) [15, 18]. SP-PCR analysis of DNA from tissues of an 18-week fetus, homozygous for E alleles, revealed a remarkably low level of instability in all tissues tested (Fig. 1A; Table 1). Conventional PCR showed homozygous E alleles of the same size (620 / 620 triplets in blood), which therefore allowed accurate determination of the magnitude of allelic size variation. Analysis of 2320 individual *FXN* molecules from multiple tissues revealed a cumulative mutation load of 4.2% (Table 1). Compared to a 24 year-old FRDA patient, homozygous for two E alleles of the same size (943 / 943 by conventional PCR analysis), fetal tissues had 7.3-fold less instability than the corresponding adult-derived tissues (4.2% versus 30.6%, $P<0.0001$; Fig. 1 B & C; Table 1). The overall mutation load is skewed in favor of contractions (3 to 4-fold more contractions) in both adult and fetal tissues (Table 1). Analysis of an additional 572 molecules from a primary fibroblast cell line derived from the fetus also revealed similarly low levels of instability (2.1%). Blood and cerebellum showed the highest mutation load. Interestingly, these tissues also revealed the highest frequency of large mutations (+ / - 20% of the progenitor allele length), which amounted

to 15% and 31% of all mutations in blood and cerebellum, respectively (examples are indicated by asterisks in Fig. 1A). Together, these data indicate that the relatively high level of somatic instability observed in adult-derived tissues develops after early embryonic development.

It is known that non-GAA interruptions within an E allele can stabilize the sequence in somatic cells *in vivo* [17]. To rule out the possibility that similar intra-allelic interruptions may be the reason for the stability seen in fetal tissues, we analyzed blood samples from the parents of the fetus to test if the inherited E alleles were capable of developing somatic instability. In contrast to the fetal sample, SP-PCR analysis of ~250 *FXN* molecules derived from both of the heterozygous parents showed significantly higher mutation loads (61.5% [maternal], 49.8% [paternal] versus 7.5% [fetal]; $P<0.0001$ in both cases; Fig. 2B). This difference is unlikely to be due to the length of the E alleles since the maternal E allele (680 triplets) was similar in size to the fetal E alleles (620 triplets). This indicates that the fetal E alleles are indeed capable of developing somatic instability, but are nevertheless stable due to the younger age.

Somatic instability in FRDA progresses throughout life

SP-PCR analysis of 6288 individual *FXN* molecules derived from a panel of blood samples from FRDA patients and heterozygous carriers with ages ranging from 2 – 49y (Supplementary Table 1) showed mutation loads ranging from 17% - 78.7%, and it correlated significantly with the age of the donor ($R=0.88$; $P=0.0007$). Including the blood sample from the fetus, the mutation loads ranged from 7.5% - 78.7% and showed a highly significant correlation with age ($R=0.91$; $P=0.0001$; total 6835 individual *FXN*

molecules; Fig. 3), with donor age accounting for 83% of the variability. The donors were homozygous / heterozygous for constitutional E alleles of various sizes as determined by conventional PCR (346 – 1000 triplets; Supplementary Table 1), however no statistically significant allele length effect was observed (P=0.18 & P=0.39 for the longer and shorter E alleles, respectively). These data indicate that somatic instability *in vivo* is progressive and increases throughout life.

DISCUSSION

The age-dependent increase in mutation load, and the remarkably low level of instability through early embryonic development, indicate that DNA replication is unlikely to be the only mechanism for somatic instability in FRDA patients. The age-dependent increase of mutation load in peripheral blood cells, despite their limited lifespan in the circulatory system, indicates that somatic instability develops progressively in hematopoietic progenitors, and not through the many cell divisions occurring from the progenitor cell stage through end cells in the peripheral circulation.

Our data contrast with the frequent observation of DNA instability in simple replication model systems established in *E. coli* [17] and *S. cerevisiae* [16]. However, they are consistent with the observation of progressive somatic instability of expanded $(CAG{\cdot}CTG)_n$ repeats in tissues from patients with Huntington disease [19] and myotonic dystrophy [20, 21, 22], and in transgenic mouse models [23, 24, 25]. It is noteworthy that this commonality transcends obvious differences in the physical properties of the two repeat sequences, and their genomic locations. Despite this general similarity, instability of the expanded GAA-TR sequence involves mainly contractions [18], as opposed to mainly expansions of the (CAG·CTG)n sequence. Furthermore, somatic instability of the (CAG·CTG)n sequence is thought to progress in small incremental steps [20, 21, 22], but our data from fetal tissues show that somatic instability in FRDA may include large expansions and contractions. Indeed, given the overall low level of mutation in fetal tissues, the rather high prevalence of large mutations in blood and cerebellum indicate that the latter are single mutation events and are not due to multiple incremental mutations. It remains to be seen if mammalian mismatch repair proteins, so

critical for the development of somatic instability of $(CAG{\cdot}CTG)_n$ repeats [26, 27, 28, 29], also mediate somatic instability of the GAA-TR sequence.

A potential weakness of our study is that, despite considerable effort, we were able to ascertain tissues from only one fetus homozygous for expanded GAA-TR alleles. However, the demonstration of instability in both parental alleles supports the case that the fetal alleles are inherently capable of somatic instability. Moreover, the lower instability of the 2 year-old individual (mutation load = 17.5%), and the statistically significant trend of increasing somatic instability through middle-age (49 y), support our conclusion that somatic instability in FRDA is mostly postnatal, and progresses throughout life. Another caveat of our study involved the use of multiple individuals of varying ages instead of studying a selected group of patients over time. However, the highly significant statistical correlation of mutation load with patient age, the lack of correlation with allele length, the similarity of mutation load in patients of similar ages (16y versus 17y, P=0.95; and 24y versus 29y, P=0.52), and our recent observation of age-dependent increase in somatic instability in a transgenic mouse model [30], all support the conclusion that somatic instability of expanded GAA-TR sequences at the *FXN* locus is age-dependent.

The age-dependent progression of somatic instability provides a conceptual framework for the connection between disease pathogenesis and somatic instability in FRDA. The observation that mutation load in blood increases throughout life, and the dramatic difference between multiple fetal and adult tissues suggest that it is likely a generalized phenomenon. This is likely to be the basis of the development of late onset FRDA in patients who carry one borderline allele (44 – 66 triplets) and one conventional

E allele (66 – 1700 triplets). Borderline alleles are thought to be too short to impede *FXN* gene transcription, but accumulation of expansions via somatic instability is thought to underlie the development of the FRDA phenotype [15]. Given our present results, we propose that progressive somatic instability of borderline alleles results in late onset FRDA. Moreover, the age-dependent accumulation of large expansions in DRG of FRDA patients [14] is further supported by our current observation of progressive somatic instability in human tissues.

In summary, the expanded GAA triplet-repeat sequence displays age-dependent increase of mutation load in somatic cells *in vivo*. Progressive somatic instability may contribute to disease pathogenesis in Friedreich ataxia.

MATERIALS AND METHODS

Tissue samples: Samples from pediatric and adult FRDA patients and carriers was collected using institutionally approved protocols at OUHSC and INNN. Autopsy material from the 18-week fetus, homozygous for expanded GAA triplet-repeat sequences in the *FXN* gene, was obtained from the "Telethon Bank of Fetal Biological Samples" in the Facoltá di Medicina e Chirurgia, University of Naples "Federico II", Italy (Director: Prof. Lucio Nitsch; funded by Telethon Italia). Pediatric and adult tissues were processed and analyzed in the Bidichandani lab in Oklahoma (USA). All fetal tissues were processed and analyzed in the Cocozza lab in Naples (Italy).

Small pool PCR analysis: This was performed as described previously [18, 31]. Briefly, serial dilutions of human genomic DNA (starting in the range of 6 – 600 pg) were prepared in siliconized microfuge tubes. PCR was performed using primers 147F (5'-GAAGAAACTTTGGGATTGGTTGC-3') and 602R (5'-AGGACCATCATGGCCACACTT-3'). PCR products were detected by Southern blotting using an end-labeled $(TTC)_9$ oligonucleotide probe. The calculation of the number of individual molecules per reaction was performed by Poisson analysis as described previously [18, 31]. For each genomic DNA sample, multiple reactions were performed using "small pools" of 10 – 25 individual molecules per reaction to detect mutations. Mutation load was calculated as the proportion of amplified *FXN* molecules that differed by >5% in length from the constitutional (most common) allele, as determined by conventional PCR.

Statistical analysis: Proportions were compared using the Chi square test, and correlation of instability with age / allele length was calculated using Pearson's coefficient of correlation.

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FIGURE LEGENDS

Figure 1: SP-PCR showing significantly low levels of somatic instability in fetal tissues. **(A & B)** Southern blots showing limited somatic variability in representative tissues of an 18-week fetus (A) and a 24y adult patient (B), each of whom are homozygous for E alleles. Each lane contains "small pools" of 12 – 20 individual *FXN* molecules. Arrowheads indicate the position of the constitutional allele determined by conventional PCR. Asterisks indicate rare instances of large expansions/contractions despite the overall low level of somatic instability. DNA size markers are shown as dashes along the right margin, which indicate the relative positions of 1 kb (182 triplets), 2 kb (515 triplets), 3 kb (849 triplets) & 4 kb (1182 triplets). **(C)** Cumulative average mutation load of all fetal versus adult (patient A-1) tissues analyzed showing a highly significant, 7.3-fold lower level of somatic instability in fetal tissues compared with adult tissues. Error bars depict +/- 2SEM.

Figure 2: SP-PCR showing instability of alleles inherited by the fetus in both heterozygous parents. **(A)** Representative SP-PCR results showing high levels of somatic instability of the E alleles in blood samples from the heterozygous carrier parents of the fetus (the arrowhead indicates the position of the single E allele in either parental blood sample as determined by conventional PCR; normal alleles are not shown). This indicates that both E alleles that were transmitted to the homozygous offspring are inherently capable of somatic instability. Each lane contains "small pools" of 17 – 20 individual *FXN* molecules. DNA size markers are shown as dashes along the right margin, which indicate the relative positions of 1 kb (182 triplets), 2 kb (515

triplets), 3 kb (849 triplets) & 4 kb (1182 triplets). **(B)** Bar graph of mutation load in the blood of fetus versus both parents (combined) showing a highly significant, 7-fold lower level of somatic instability in fetal tissues compared with adult tissues. The error bar depicts +/- 2SEM.

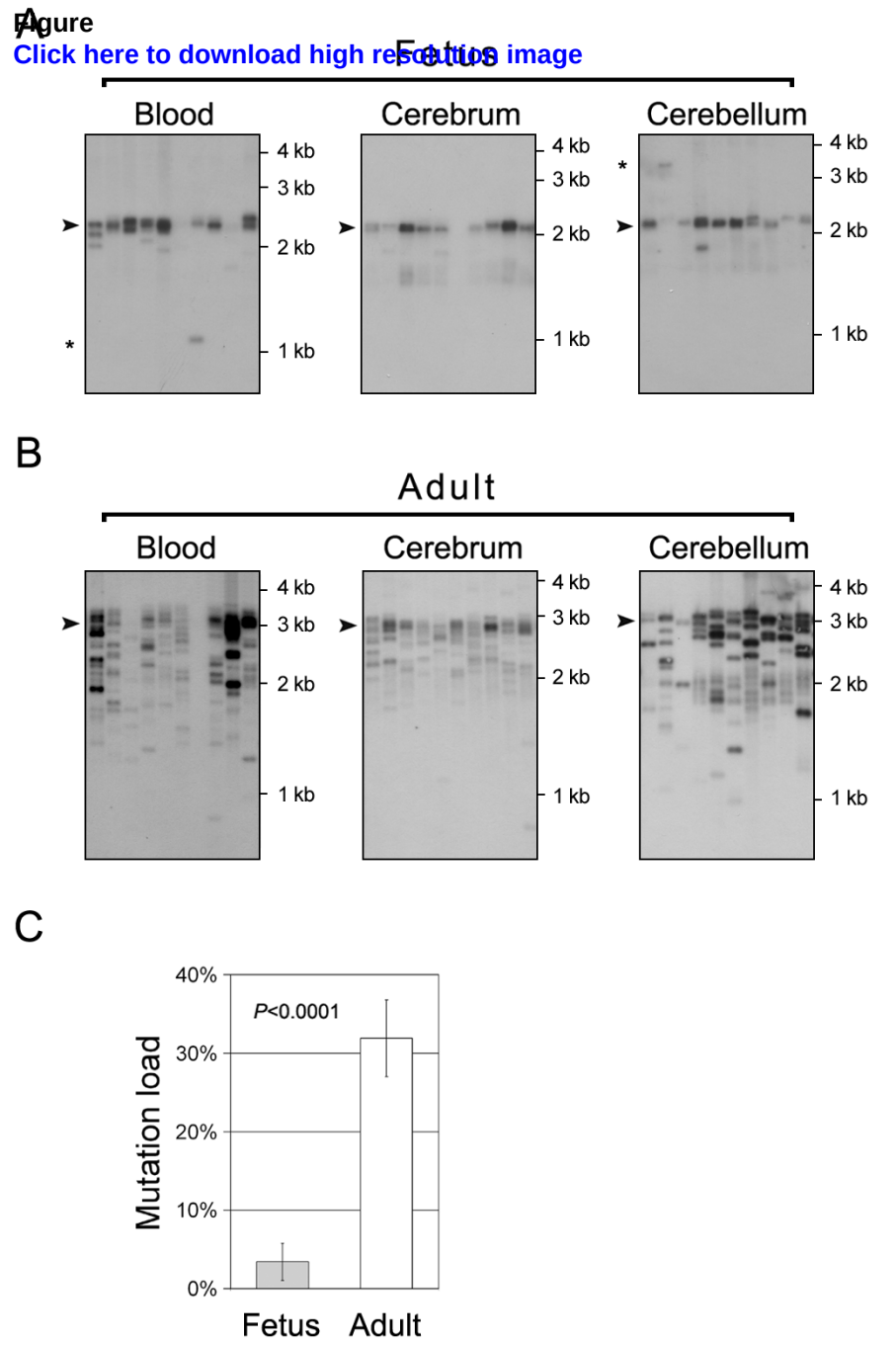
Figure 3: Age-dependent increase of somatic instability in FRDA patients and carriers. Mutation load (Y-axis) is plotted against age (in years; X-axis) for blood samples derived from ten individuals. Their ages include 18-weeks gestation (plotted as 0y), 2y, 9y, 16y, 17y, 24y, 29y, 36y, 43y, and 49y. Error bars indicate 95% confidence intervals.

Table

	Fetus				Adult[1]				
Tissues	Molecules	Mutation load	Contractions	Expansions	Molecules	Mutation load	Contractions	Expansions	*P* value[2]
Blood	548	41 (7.5%)	29 (5.2%)	12 (2.1%)	1347	567 (42.1%)	524 (38.9%)	43 (3.2%)	<0.0001
Cerebrum	873	14 (1.6%)	13 (1.5%)	1 (0.1%)	1249	274 (21.9%)	215 (17.2%)	59 (4.7%)	<0.0001
Cerebellum	483	34 (7%)	25 (5.2%)	9 (1.9%)	1008	273 (27.1%)	212 (21%)	61 (6.1%)	<0.0001
Spinal Cord	156	1 (0.6%)	1 (0.6%)	0 (0%)	269	99 (36.8%)	80 (29.7%)	19 (7.1%)	<0.0001
Heart	156	6 (3.8%)	4 (2.6%)	2 (1.2%)	711	193 (27.1%)	113 (15.8%)	80 (11.3%)	<0.0001
Pancreas	104	1 (1%)	1 (1%)	0 (0%)	619	187 (30.2%)	114 (18.4%)	73 (11.8%)	<0.0001
Total	2320	97 (4.2%)	73 (3.1%)	24 (1%)	5203	1593 (30.6%)	1258 (24.2%)	335 (6.4%)	<0.0001

[1]Autopsy of a 24 year old FRDA patient; [2]Comparison of mutation load

Figure



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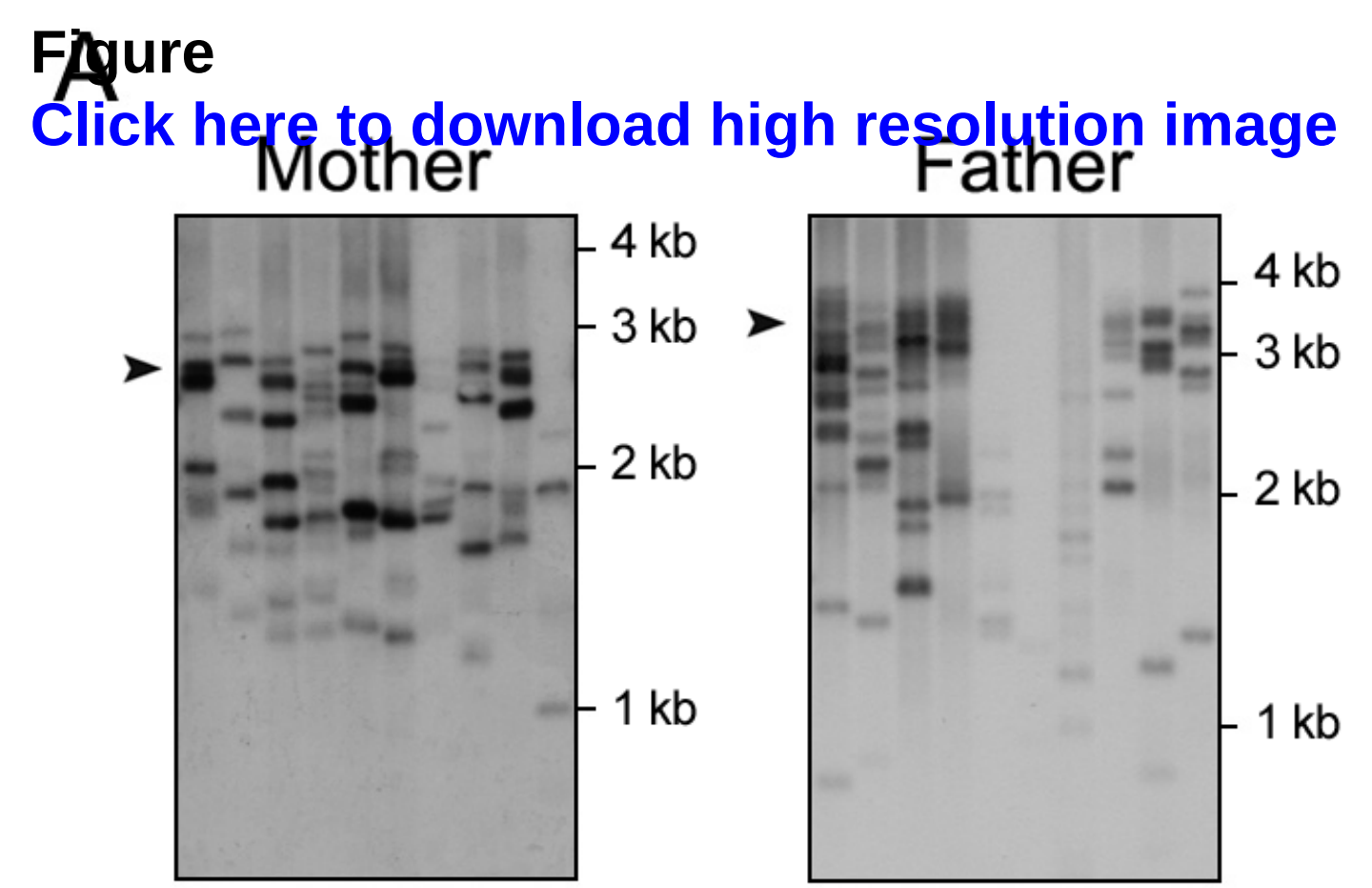

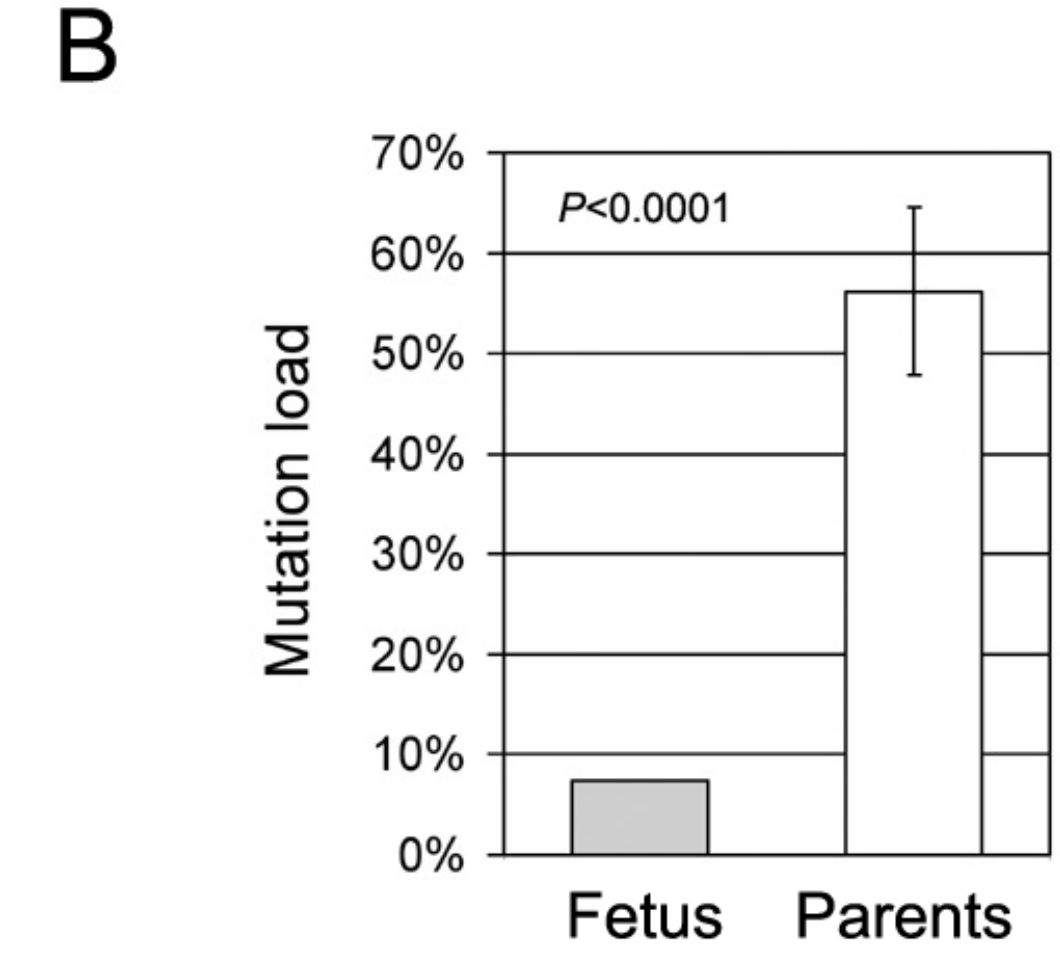

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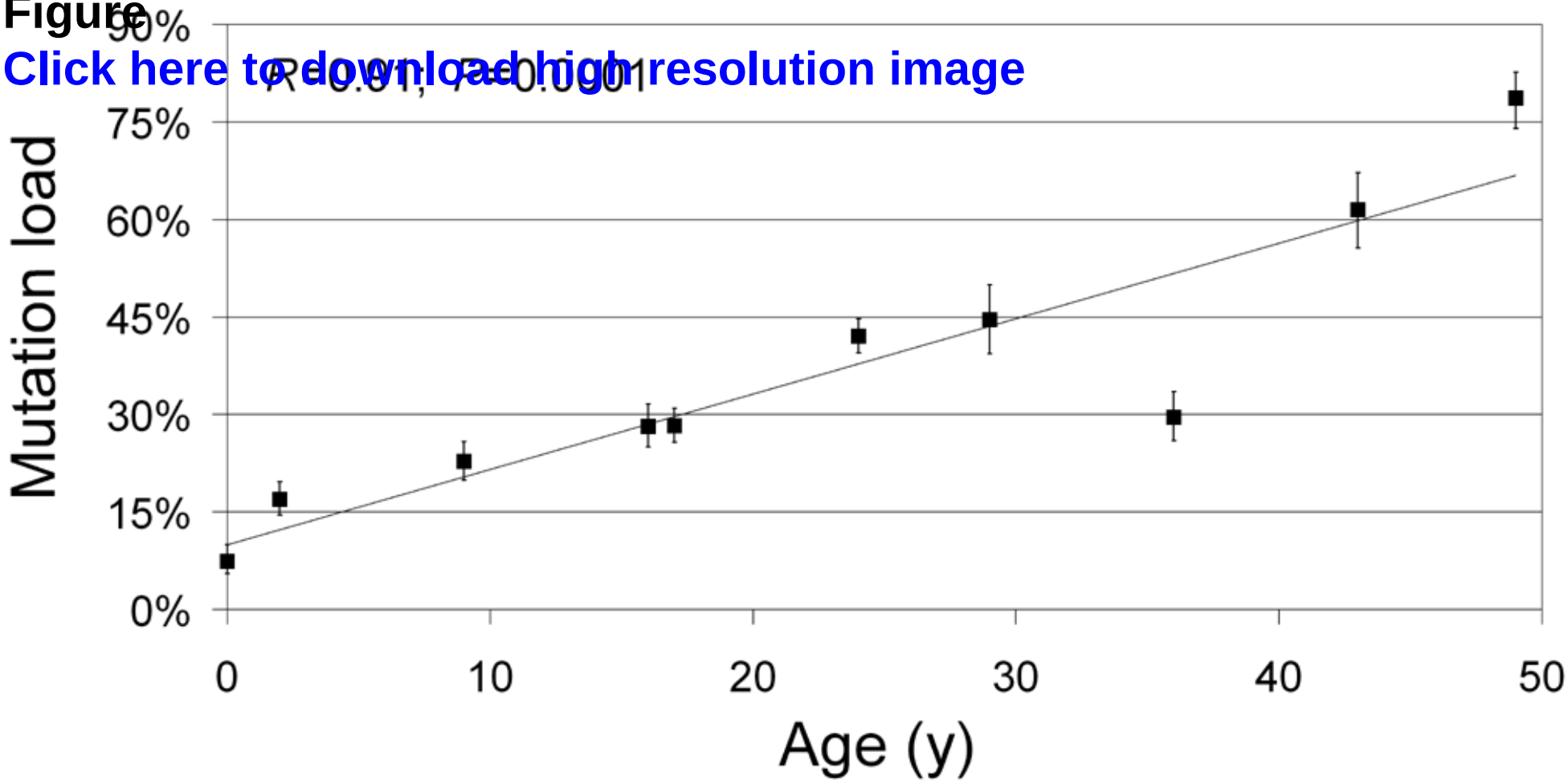

Supplemental Information (Table of mutational data)

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