***Drosophila* mitochondrial transcription factor A (*d*-TFAM) is dispensable for the transcription of mitochondrial DNA in Kc167 cells**

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We have cloned cDNA encoding *Drosophila* mitochondrial (mt) transcription factor A (*d*-TFAM). RNA interference (RNAi) of *d*-TFAM by lipofection of haemocyte-derived Kc167 cells with double-stranded RNA reduced *d*-TFAM to less than 5% of the normal level. Reflecting the ability of TFAM to stabilize mtDNA, RNAi of *d*-TFAM reduced mtDNA to 40%. Nonetheless,

transcription of the *ND2* and *ND5* genes and their mRNAs remained unchanged for 8 days of the duration of RNAi. We thus show that *d*-TFAM is not essential for the transcription of *Drosophila* mtDNA.

Key words: HMG box, mitochondria, RNA interference.

INTRODUCTION

Human mitochondrial (mt) transcription factor A (*h*-TFAM; formerly referred to as *h*-mtTFA) is a factor of 29 kDa identified based on transcriptional activation of human mtDNA *in vitro*, and has two HMG boxes with a linker between them and a C-terminal tail sequence [1–3]. Many putative binding sites of *h*-TFAM have been demonstrated in the control region of human mtDNA, which contains heavy- (H-) and light- (L-) strand transcription start sites [4]. Homozygotic knockout of the mouse gene (*m-tfam*) resulted in severe mtDNA depletion and embryonic death on day 10.5, and heterozygotic knockout exhibited reduced mtDNA levels and respiratory chain deficiency, mainly in heart [5]. The yeast (*Saccharomyces cerevisiae*) homologue, *sc*-mtTFA, is an abundant protein composed almost entirely of two HMG boxes separated by a short linker [6]. *sc*-mtTFA was originally described as ABF2 that could bind to the replication origin of yeast nuclear DNA, but was later found to be localized in mitochondria [7]. Disruption of the *abf2* gene led to a loss of respiratory competence and mtDNA when cells were grown in the presence of glucose. Since expression of *h*-TFAM in the yeast *abf2* strain improved the phenotype, a potential functional homology of *h*-TFAM with *sc*-mtTFA was confirmed [8]. *Xenopus* mtTFA was also cloned and found to stimulate the transcription of *Xenopus* mtDNA *in vitro* up to 10-fold for the H-strand, but 3-fold for the light L-strand [9].

Despite these lines of evidence for the essential role of TFAM, another factor has been suggested for the transcription of yeast and *Xenopus* mtDNA. In yeast, a core RNA polymerase of 145 kDa with sequence similarity to viral RNA polymerases of T3, T7 and SP6 [10] initiates the transcription together with a dissociable 39 kDa mt transcription factor B (*sc*-mtTFB) [11,12]. These two proteins bind co-operatively to a nonanucleotide promoter sequence [11,13] present at multiple locations in the 75 kb yeast mt genome [14]. *Xenopus laevis* (*x*-) mtTFB (no evidence for homology with *sc*-mtTFB) has also been characterized [15] and it alone allows RNA polymerase to localize and

initiate transcription from a minimal octanucleotide promoter encompassing the transcription start sites [15–17].

As with many other nuclear proteins having an HMG box, vertebrates TFAMs and the yeast homologue (*sc*-mtTFA) have the ability to bend DNA and to wrap around distorted DNA with low nucleotide-sequence specificity [6,9,18,19]. It is possible that transcriptional activation of mtDNA by TFAMs is due to such interaction with DNA distinct from the guidance of RNA polymerase to the promoter sequences.

We have cloned *Drosophila* TFAM (*d*-TFAM) and analysed its function by RNA interference (RNAi) in *Drosophila* haemocyte-derived Kc167 cells. We show that lipofection of double-stranded (ds) RNA encoding the sequence of *d-tfam* depletes *d*-TFAM almost completely. A consequence of the depletion was a marked reduction of mtDNA levels while mt mRNA levels remained unchanged.

MATERIALS AND METHODS

Screening and sequencing of *d-tfam* cDNA clones

The amino acid sequences of *h*-TFAM and *x*-TFAM were subjected to homology searching using the TBLASTN algorithm in the Berkeley *Drosophila* Genome Projects database. A fragment of cDNA encoding a sequence that was highly homologous with both *h*-TFAM and *x*-TFAM was found in the EST database. To isolate the full-length cDNA, a cDNA fragment was prepared by reverse transcriptase PCR of RNA extracted from Kc167 cells using paired primers, 5'-AGCTTACAACGCAAGGGC-3' and 5'-GTGAATTATGTGATGGAAGAG-3', based on the EST sequence. A *Drosophila melanogaster* cDNA library of $\approx 5 \times 10^5$ phage constructed from mRNAs from adult male and female flies (Stratagene) was screened in a buffer containing 50 mM Tris/HCl (pH 7.5), $5 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), $5 \times$ Denhardt's solution, 1% SDS and 0.1 mg/ml salmon sperm DNA. After hybridization for 16 h at 65 °C, filters were washed three times with $2 \times$ SSC containing 0.1% SDS at room temperature and once with $0.2 \times$ SSC

Abbreviations used: ds, double stranded; mt, mitochondrial; TFAM, mt transcription factor A; *h*-TFAM, human TFAM; *d*-TFAM, *Drosophila* TFAM; *sc*-, *Saccharomyces cerevisiae*; *x*-, *Xenopus laevis*; mtTFB, mt transcription factor B; RNAi, RNA interference; H-strand, heavy strand; L-strand, light strand.

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containing 0.1 % SDS at 65 °C for 30 min, and exposed to X-ray film (Fuji). cDNA was sequenced by the dideoxynucleotide method with an Applied Biosystems model 310 DNA sequencer.

Preparation of anti-*d*-TFAM antiserum

To express *d*-TFAM in *Escherichia coli*, a PCR fragment of *d-tfam* cDNA encoding the amino acid sequence from Glu-42 to Ile-257 was cloned into the *Bam*HI-*Sal*II sites of pGEX-2T (Amersham Pharmacia Biotech). Bacterial culture harbouring the plasmid was grown in LB medium containing 100 µg/ml ampicillin, and the expression was induced by 0.1 mM isopropyl β-D-thiogalactoside. The glutathione S-transferase fusion protein was purified using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. New Zealand white rabbits were immunized by intramuscular injections of the purified protein (0.3 mg) in complete Freund's adjuvant and boosted twice with the protein (0.3 mg) in incomplete Freund's adjuvant. Serum was collected 6 weeks after the initial injection.

Cell culture and lipofection of dsRNA

Drosophila embryonic cell line Kc167 was maintained in HyQ CCM3 (HyClone Lab) serum-free medium at 25 °C. The cells were plated at a density of 2.0×10^6 cells per 6-well Multidish (Nunc) and lipofected with 6 µg of *d-tfam* (nt 119–892) or *odds-paired* (*opa*; nt 103–1124) dsRNA with the aid of a liposome reagent kit (Qiagen). One-fifth of each culture was removed every other day to obtain cells for further analyses.

Preparation of dsRNA

The templates for the production of dsRNA were PCR-derived fragments sandwiched by double T7 promoter sequences. For the production of sense or antisense single-stranded RNA, PCR templates sandwiched by T3 and T7 sequences were transcribed with T3 or T7 RNA polymerase (Stratagene), respectively. After treating with RNase-free DNase, RNA products were purified by phenol/chloroform extraction and ethanol precipitation. Although RNAs derived from double T7 promoter PCR templates self-annealed during synthesis, the annealing was completed by dissolving precipitated RNAs in annealing buffer (2 mM Tris/HCl, pH 7.5/2 mM EDTA), boiling for 2 min and keeping at room temperature for 16 h.

Western blotting

The lipofected cells were lysed in a buffer containing 10 mM Tris/HCl, pH 7.5, 2 mM EDTA and 1 % SDS. Cell lysate corresponding to 10 µg of protein was separated by SDS/PAGE under reducing conditions using 12 % polyacrylamide gel, and proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with PBS containing 5 % skimmed milk for 1 h, reacted with 500-fold diluted antiserum against *d*-TFAM for 16 h at 4 °C, washed extensively and reacted with 1000-fold diluted horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. The ECL[®] Western-blotting system (Amersham Pharmacia Biotech) was used for the detection. Polyclonal antibody against *Drosophila* laminin β-chain was obtained and used as described in [20].

Indirect fluorescence immunostaining of Kc167 cells

After labelling with MitoTracker Red CMXRos (Molecular Probes) according to the manufacturer's instructions, Kc167 cells were fixed with methanol/water/acetic acid (95:4:1, by

vol.) for 15 min. They were then permeabilized with cold methanol for 10 min, incubated with antiserum against *d*-TFAM (1:500 in buffer A: PBS containing 0.1 % Tween 20) for 1 h, washed four times with buffer A, incubated with FITC-conjugated anti-rabbit IgG (Cappel; 1:500 in buffer A) for 1 h and washed four times with PBS. They were then mounted in 80 % (v/v) glycerol containing 2 mM Tris/HCl (pH 8.0)/0.2 M 1,4-diazobicyclo[2.2.2]octane.

Northern and Southern blotting

Total RNA was extracted from the lipofected cells by TRIzol reagent (Gibco-BRL). RNA corresponding to 10 µg was separated in a 1.2 % agarose/formaldehyde gel, blotted on to Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) and hybridized to ³²P-labelled *Rp49*, *ND2* (nt 288–1045), *ND5* (nt 6516–8053), mt rRNAs (mt 16 S and 12 S rRNA; nt 12824–14756) or *d-tfam* (nt 119–892) at 42 °C in 5 × SSPE (standard saline phosphate/EDTA, 150 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.4), 0.5 % SDS, 5 × Denhardt's solution, 50 % formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1 % SDS at room temperature and twice with 0.1 × SSC containing 0.1 % SDS at 55 °C for 30 min, and then analysed by a Fuji Film BAS 2000 Image Analyzer. Genomic DNA was extracted by DNAzol reagent (Gibco-BRL). DNAs corresponding to 10 µg of extracted samples were digested with *Pst*I and *Hind*III (Toyobo), electrophoresed in a 1 % agarose/TAE (Tris/acetate/EDTA) gel, blotted to Hybond-N⁺ nylon membrane and hybridized to ³²P-labelled *ND5* (nt 6516–8053) or *hml* (DDBJ/EMBL/GenBank accession number AB035891; nt 6532–7260) at 65 °C in 50 mM Tris/HCl, pH 7.5/1 % SDS/5 × SSC/5 × Denhardt's solution/0.1 mg/ml of salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1 % SDS at 65 °C and twice with 0.2 × SSC containing 0.1 % SDS at 65 °C for 30 min.

Assay of *in vivo* transcription activity of mtDNA

After lipofection of dsRNA (4 days), Kc167 corresponding to 8×10^6 cells were rinsed twice with 10 mM Hepes buffer, pH 6.8, containing 140 mM NaCl and radiolabelled by incubating in 1.1 ml of the same buffer containing 1 mCi of [³²P]P_i (Amersham Pharmacia Biotech) for 2 h. Total RNAs were extracted by TRIzol reagent and the labelled RNA corresponding to 1×10^7 c.p.m. was hybridized to 2 µg of Bluescript vector, *ND2*, *ND5* or mt rRNAs fragments which were pre-alkali treated, neutralized and dotted on to Hybond-N⁺ nylon membrane. The hybridization was carried out at 42 °C in a buffer containing 5 × SSPE, 0.5 % SDS, 5 × Denhardt's solution, 50 % formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1 % SDS at room temperature and twice with 0.1 × SSC containing 0.1 % SDS at 55 °C for 30 min, and analysed by a Fuji Film BAS 2000 Image Analyzer.

RESULTS

Cloning of *d-tfam*

A BLAST search of the Berkeley *Drosophila* Genome Projects database yielded an EST fragment encoding an amino acid sequence homologous with *x*- and *h*-TFAMs. The full-length cDNA had an open reading frame encoding the 257-amino-acid sequence, shown aligned in Figure 1 with those of *x*- [9], *h*- [3] and *m*-TFAMs [21]. The PSORT II algorithm [22] predicts the

	<u>N-terminal region:</u>		
fly	MI-----YTTLMSSRGGLIGSLINKVRPLAAASISNTPAVPSITLEEQLGL	47	
frog	MRQTGSLALVARAGQKDRVLSLPLVMMSLMRGVGGLLRSLTGNCSTIRCTNVSSGIFGFQCSPLRCFSKGAAP	77	
human	MA-----FLRSMWGLSALCRSAELCTGCGSRLRPFPSFVYLP-RWFSSVL--	46	
mouse	MA-----LFRGMWSVLKALRTQVEMCAGCGGRIPSSISLVCIP--CFSSM---	45	
	<u>HMG box 1:</u>		
fly	PPRPKKPTPTPRMREORPKLKAANPQITVEVVRQLSKNMSDADAQLKERLQAEFRDOOIVVEERTKY	118	
frog	SDYPKRPISGYLRYSEORPKLHKQYPEAKMMDLTKIIALEKGLASTEKEPYEVAADLKYYEEVKQY	148	
human	ASCPKKPVSSYLRFSEKQIPFKAQNPDAKTELIRIAQRRELPSKKKIYDAYRAEWVYKEISRF	117	
mouse	GSYPKKPMSSYLRFSTEQLPKPKAKHPDAKLSLVKIAALRELPEAEKKVYEADFAEWKAYKEAVSKY	116	
	<u>linker region:</u>		
fly	DATLDEEORAEIKQLKQLVDKERRQLRR-VKE	152	
frog	REALSPVQLELHREQRORRLAKRKSVRKKRELTA	182	
human	KEQLPSQIMSLEKEIMKH-LRRKAMTKKELTL	151	
mouse	KEQLPSQIMGMEKEARQR-LKKKALVKRELIL	150	
	<u>HMG box 2:</u>		
fly	LGRPKKPASAPLRIASERINTPOGDKQTYREWHQKTTAKMRLSDSEKEVVMESRKEMELRKAISSV	222	
frog	LGRPKRPSPPNIMSEHFQDAKCTSSQTK--MKSLRDEMERHNTOKOTYNHQAQDDKIYENEMKSW	249	
human	LCKPKRPSAYNVYV-AERFQEAAGDSPOEK--LKTIVENMKNLSDSEKELVIOHAKEDETRYHNEMKSW	218	
mouse	LCKPKRPSAYNIYV-SEFQEAQDSAGGK--LKLVNEAMKNLSPEEKQAMVIOAKDDRIYDNEMKSW	217	
	<u>C-terminal tail:</u>		
fly	EBMMRLCHIDVVR-----HGNLIDPPEPKPRVTLASKDI	257	
frog	EBOMIEIGRGLIRLNQRKRFKKPRATRASSKNTAKKGLTGSSSHKEGFGASNPQHEE	309	
human	EBOMIEVGRKDLR-----RTIKKQRYGAE-----EC	246	
mouse	EBOMAEVGRDLIR-----RSVK--RSGDIS-----EH	243	

Figure 1 Alignment of *d*-TFAM with other TFAMs

The N-terminal signal sequence, two HMG boxes, linker and C-terminal regions of the deduced amino acid sequence of *d*-TFAM are compared with those of TFAMs of other species. Conserved residues among the four species are printed on a black background. Other conserved residues are highlighted with a grey background.

Figure 2 Effect of RNAi on *d*-TFAM level in Kc167 cells

Kc167 cells were either not lipofected (cont) or lipofected with *odds-paired* (opa) or *d-tfam* (ds) dsRNA. Cell lysate (10 µg) was subjected 4 days later to SDS/PAGE using 12% (upper panel) or 4% (lower panel) polyacrylamide gel and blotted with rabbit serum antibody against *d*-TFAM or *Drosophila* laminin β-chain (lanβ), respectively.

N-terminal 41 residues to be a mt targeting signal, which is followed by HMG box 1 (Pro-48–Tyr-118), a linker region (Asp-119–Glu-152), HMG box 2 (Leu-153–Trp-222) and a C-terminal

Figure 3 Indirect fluorescence immunostaining of Kc167 cells after RNAi of *d*-TFAM

Kc167 cells 4 days after lipofection with *odds-paired* dsRNA (C and D) or *d-tfam* (E and F) dsDNA, and control cells (A and B), were double-stained with rabbit antiserum against *d*-TFAM/FITC-conjugated goat antibody against rabbit IgG and dye for living mitochondria (MitoTracker). Fluorescence micrographs were taken through a FITC filter (A, C and E) or a Rhodamine filter (B, D and F).

tail (Glu-223–Ile-257). Overall similarity of the sequence to the *x*-, *h*- and *m*-TFAM sequences was 45, 36 and 39%, respectively. The sequence predicts sizes of 29961 and 25748 kDa and pI

Figure 4 Effect of RNAi on the level of mtDNA

Total DNA (10 µg) of control Kc167 cells (cont) or Kc167 cells 4 days after lipofection with *odds-paired* (*opa*) or *d-tfam* (*ds*) dsRNA was digested with *Pst*I and *Hind*III restriction enzymes, separated in agarose/TAE gel, blotted on to nylon membrane and hybridized with radiolabelled *ND5* (mtDNA) or *hml* (control) probes.

values of 10.41 and 10.28 for the full-size and targeting-signal-clipped forms, respectively. Antiserum raised against a recombinant protein of Glu-41–Ile-257 detected, by Western blotting of Kc167 cell lysate, a weak band and strong doublet bands migrating within the range of 34–29 kDa (Figure 2, upper panel,

control). Relatively slower migration in SDS/PAGE may be due to high content of basic amino acids. Whereas the weak band can be attributed to the precursor form before localization in mitochondria, the reason for detecting the doublet bands is unknown. Two alternatively spliced transcripts of *m-tfam* were found to produce *m-TFAM* and a testis-specific nuclear HMG-box protein lacking the mt targeting signal [21], but indirect fluorescence immunostaining of Kc167 cells with the antiserum showed signals that overlap considerably with dye that stains living mitochondria (MitoTracker; Figure 3).

Suppressed level of *d*-TFAM by lipofection of Kc167 cells with *d-tfam* dsRNA

Lipofection of Kc167 cells with *d-tfam* dsRNA specifically suppressed both of the doublet bands of *d*-TFAM. Figure 2 depicts Western blotting of the cell lysate 4 days after the lipofection. The signal for the doublet bands was decreased to one-twentieth whereas the signal for laminin β -chain, used as an internal control, remained unchanged. The effect was sequence-specific since lipofection of dsRNA encoding *odds-paired* (*opa*) showed no effect. Lipofection of single-stranded sense or anti-sense RNA encoding *d-tfam* showed no effect (results not shown). When the time course was followed by preparing cell lysate every other day, the suppression was evident 2 days after the lipofection and continued at least until the eighth day, when the effect started to be cancelled (results not shown).

Immunostaining of the cultures confirmed efficient RNAi of *d*-TFAM (Figure 3). Whereas most cells in control (Figure 3A) and *odds-paired* dsRNA-lipofected (Figure 3C) cultures were positive to *d*-TFAM staining, the signal disappeared from almost all cells in *d-tfam* dsRNA-lipofected culture (Figure 3E). Despite marked reduction of *d*-TFAM levels, however, the signal for living mitochondria remained unchanged (Figure 3F). This suggested that mt membrane potential was retained even under suppression of the *d*-TFAM level. Consistent with this observation, the cells lipofected with *d-tfam* dsRNA grew at a rate comparable with the control cells (results not shown).

Figure 5 Effect of RNAi on the level of mtDNA transcripts

Total RNA (10 µg) of control Kc167 cells (cont) or Kc167 cells 6 days after lipofection with *odds-paired* (*opa*) or *d-tfam* (*ds*) dsRNA was separated in 1.2% agarose/formaldehyde gel, blotted on to nylon membrane and hybridized with radiolabelled *d-tfam*, mt rRNAs, *ND5*, *ND2* or *Rp49* probes. The *Rp49* probe was used as an internal control for the genes encoded by the nuclear genome.

Figure 6 Effect of RNAi on the transcription activity of mtDNA

Bluescript vector (BS), ND2, ND5 and mt rRNA fragments (2 µg each) were dotted as duplicates on nylon membrane and hybridized with 1×10^7 c.p.m. of total RNA extracted from control cells or cells lipofected with *d-tfam* dsRNA (dsdTFAM). The photostimulated luminescence (PLS) count for each spot was calculated by a Fuji Film Image Gauge version 3.4.

Reduction of mtDNA level while mt mRNA levels were unchanged

Figure 4 depicts the effect of *d*-TFAM depletion on the level of mtDNA. When normalized with the same amount of total cellular DNA, a Southern blot probed with the ND5 cDNA sequence showed a reduction of mtDNA to 40% in the cells 4 days after lipofection of dsRNA. This suggests destabilization of mtDNA in the absence of *d*-TFAM, probably due to its ability to wrap around distorted DNA. This level of 40% mtDNA was not transient because essentially the same result was obtained for the cells 6 days after lipofection (results not shown).

Consistent with no apparent change of phenotype in the mt membrane potential and the proliferation activity, the level of mt mRNAs remained unchanged in the *d*-TFAM-depleted cells, as monitored by Northern blot of ND2 and ND5 mRNAs (Figure 5). The result showed that *d*-TFAM is essential for the transcription of neither the H-strand (ND2) nor L-strand (ND5) of *Drosophila* mtDNA. Slight reduction of 12 S and 16 S rRNAs might suggest a role for *d*-TFAM in the transcription of rRNA genes, but it is more likely due to the reduction of gene dosage.

Since it is well known that mt RNA is rather stable, the unchanged level of mt mRNAs observed by Northern blotting could be simply due to their slow turnover. In order to address this question, newly synthesized transcripts were measured by pulse-labelling of the cells with [32 P]P_i and hybridization of radioactive RNA with DNA probes dotted on to nylon membrane (Figure 6). The radioactivity hybridized to ND2 and ND5 probes was virtually the same in control and dsRNA-lipofected cells. This result confirmed that *d*-TFAM is dispensable for the transcription of *Drosophila* mt mRNAs. On the other hand, the radioactivity hybridized to the mt rRNA probe was decreased to half in the *d-tfam* dsRNA-lipofected cells.

DISCUSSION

Suppression of *d*-TFAM protein to less than 5% of the normal level by RNAi did not affect transcription of ND2 and ND5 genes and their mRNAs remained unchanged during the period

that it took the Kc167 cells to double 10 times (8 days). The membrane potential across mt inner membrane was also not affected. Our results thus show that *d*-TFAM is not essential for transcription of *Drosophila* mtDNA. Since the properties of TFAMs may vary between species; however, we need to be careful in using the data to generalize about other TFAMs.

As a member of the HMG-box proteins, TFAM is implicated to stabilize mtDNA by wrapping around and condensing DNA [6,9,18]. Probably reflecting the same activity of *d*-TFAM, *d*-TFAM depletion in Kc167 cells resulted in reduced mtDNA levels. An evident phenotype of disrupting *m-tfam* in mice was mtDNA depletion, which was accompanied by respiratory chain deficiency [5]. It was possible that the mtDNA depletion was the direct consequence of *m-tfam* disruption and that the respiratory chain deficiency was an indirect consequence caused by reduction of the gene dosage.

Despite no apparent effect on mt mRNAs, *d*-TFAM depletion reduced the transcription of mt rRNA genes. To support the translation of all mt mRNAs, mt rRNAs are needed in large excess of mt mRNAs. Studies on HeLa cells indicated that the mechanism of H-strand transcription involves two overlapping, independently controlled transcription units starting at closely located sites, one covering the rRNA genes and the other covering the whole H-strand [23]. The differential activity of the two H-strand transcription units and an H-strand transcription attenuation event at the 3'-end of the 16 S rRNA gene account for the transcription of mt rRNAs at a rate 50 times higher than downstream mRNA and tRNA genes [24]. Although nothing is known about the transcription of insect mt rRNAs, our result suggests that such a mechanism of mt rRNA transcription specified in insects is sensitive to gene dosage (mtDNA level).

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