

# **Over-expression of *Tfam* improves the mitochondrial disease phenotypes in a mouse model system**

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## Abstract

The phenotypes of mitochondrial diseases caused by mutations in mitochondrial DNA (mtDNA) have been proposed to be strictly regulated by the proportion of wild-type and pathogenically mutated mtDNAs. More specifically, it is thought that the onset of the disease phenotype occurs when cells cannot maintain the proper mitochondrial function because of an over-abundance of pathological mtDNA. Therapies that cause a decrease in the pathogenic mtDNA population have been proposed as a treatment for mitochondrial diseases, but these therapies are difficult to apply in practice. In this report, we present a novel concept: to improve mitochondrial disease phenotypes via an increase in the absolute copy number of the wild-type mtDNA population in pathogenic cells even when the relative proportion of mtDNA genotypes remains unchanged. We have succeeded in ameliorating the typical symptoms of mitochondrial disease in a model mouse line by the over-expression of the mitochondrial transcription factor A (*Tfam*) followed by an increase of the mtDNA copy number. This new concept should lead to the development of a novel therapeutic treatment for mitochondrial diseases.

## Keywords

mitochondrial diseases, mitochondrial DNA, *Tfam*, mito-mouse

**Abbreviations:** mtDNA, mitochondrial DNA; *Tfam*, mitochondrial transcription factor A; mito-mouse, mitochondrial diseases model mouse; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy with ragged red fibers; KSS, Kearns-Sayre syndrome; CPEO, chronic progressive external ophthalmoplegia; MDS, mtDNA depletion syndrome; PGC-1 $\alpha$ , peroxisome proliferative-activated receptor  $\gamma$  coactivator-1 $\alpha$

## Introduction

Mitochondria play several essential roles in cell functions, and the accumulation of mitochondrial DNA (mtDNA) with pathogenic mutations causes mitochondrial diseases, including mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS); myoclonus epilepsy with ragged red fibers (MERRF); Kearns-Sayre syndrome (KSS); and chronic progressive external ophthalmoplegia (CPEO) [1,2].

One general feature of mitochondrial diseases is that at least two types (wild-type and mutated) of mtDNA exist in a single cell, which is a genetic status called heteroplasmy. Several mouse models have been generated to study human mitochondrial diseases, including heteroplasmic mtDNA genetics. These mice possess mtDNAs derived from two strains [3,4] containing either point-mutated mtDNA [5,6] or mtDNA that has a large deletion [7]. These mice have been used to study mtDNA transmission, pathogenic mechanisms, and the development of an effective therapies for diseases. Effective treatment procedures for diseases have not yet been established, although several attempts have been reported, including a study in which one type of mtDNA (presumably pathogenic) was specifically digested by a mitochondrial-targeted restriction enzyme [8]. However, this treatment is difficult to apply practically to medical care because the restriction sites specific to the pathogenic mutant on the mtDNA are rare.

Mitochondrial diseases are caused by the accumulation of mtDNA with pathogenic mutations in single cells followed by the reduction of mitochondrial respiration activity. Interestingly, this reduction of activity does not simply and linearly correlate with the proportion of mutated mtDNA but is caused only when the proportion of the mutated mtDNA exceeds a certain threshold level [7,9,10]. This phenomenon is thought to be caused by the occurrence of inter-mitochondrial complementation through the mixing of genetic contents [10] due to mitochondrial dynamics, such as fusion, fission and transportation [11] that makes mitochondria function as a single cellular unit [12]. The accumulation of mutated mtDNA causes a decrease in wild-type mtDNA, which results in a reduction of genetic factors and defects in respiratory activity.

The depletion of mtDNA copy number in humans [13] and mice [14] has caused severe diseases, such as mtDNA depletion syndrome (MDS). Furthermore, the

down-regulation of mtDNA replication or transcription by knockout [15] or knockdown [16] of the *Tfam* gene, which encodes the mtDNA binding protein, results in decreased respiratory activity. These data suggest that an appropriate copy number of normal mtDNA is essential for the maintenance of mitochondrial function and that cells carrying mutated mtDNA may be rescued by the increase of wild-type mtDNA sufficient to supply normal rRNAs, tRNAs and proteins.

Based on this view, we generated transgenic mice that over-express *Tfam* to increase the total mtDNA copy number [17,18], and we mated these mice to the mitochondrial diseases model mice (mito-mice) with mutant mtDNA carrying a deletion of 4696 bp ( $\Delta$ mtDNA) [7]. The increase in total mtDNA copy number due to the over-expression of *Tfam* in the mito-mice improved the severe symptoms of mitochondrial disease exhibited by the mito-mice, resulting in a prolonged life span. From this study, we propose a novel therapeutic treatment for mitochondrial diseases. We propose that an increase in the absolute copy number of healthy mtDNA restores the normal mitochondrial function even under conditions where the proportion of pathogenic mtDNA is unchanged.

## **Materials and methods**

### **Mouse strains**

The C57BL/6J (CLEA Japan), mito-mouse [7] and *Tfam*/EGFP-Tg strains were used in this research. The Institutional Animal Experiment Committee approved all of the animal experiments.

### **Estimation of mtDNA copy number by comparative real-time PCR**

Comparative real-time PCR for estimating the copy number of mtDNA in the tissues of mito-mice and Tg-mito-mice was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) with *Gapdh* as the control for the nuclear genome copy number. The following primers were used: 5'- AAC TCA AAG GAC TTG GCG GTA CTT TAT ATC-3' and 5'- GAT GGC GGT ATA TAG GCT GAA TTA GCA AGA G-3' to amplify the region common in both the wild-type and the deleted mtDNA, and 5'-AAC GAC CCC TTC ATT GAC-3' and 5'-TCC ACG ACA TAC TCA GCA C-3' for *Gapdh* [19].

The total DNA samples purified from the tissues were diluted to a concentration of 100 pg/μl - 1000 pg/μl and amplified using an ABI Prism 7900 (Applied Biosystems). The thermal cycling conditions were as follows: 50°C for 2 min; 95°C for 15 min; and 40 cycles of 95°C for 20 sec followed by 60°C for 60 sec.

### **Estimation of ΔmtDNA proportions by real-time PCR**

Real-time PCR was used to estimate the proportions of ΔmtDNA, as previously described [20].

### **Northern blot for mitochondrial tRNA**

Total RNA was purified from homogenized tissue samples using ISOGEN (NIPPON GENE), and Northern blots were then carried out as previously described [21,22]. The DNA sequences for the specific probes were as follows: 5'- ATG TTT TTA AAC ATG GAA GCA TGA A-3' for tRNA<sup>Ser(AGY)</sup> and 5'- GTC TCC CAT CCA AGT ACT AAC CAA G-3' for 5S rRNA.

### **BUN measurement**

Blood was obtained from a tail vein, and blood urea nitrogen (BUN) concentrations were measured using a Urea N B kit (Wako Pure Chemical) as previously described [23].

### **Histochemical analyses of COX and SDH activity**

Histochemical analyses of cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) activity were carried out as previously described [7,10]. Hearts and kidneys were excised from the animals, and 10- $\mu$ m cryosections from the tissues were stained for COX and SDH activity.

### **Single-fiber PCR analysis**

Two serial 10- $\mu$ m cryosections of heart muscle were used for single-fiber PCR. One of the sections was stained for COX and SDH activity, and the COX-positive fibers were selected for PCR analysis. Another section was used for the dissection of the cytoplasm corresponding to the COX-positive fibers with an LMD6000 (Leica). The total DNA was extracted from dissected samples and used directly for PCR analysis as previously described [24]. The PCR conditions were also carried out as described above.

## Results

### ***Tfam*/EGFP over-expression increased the amount of mtDNA**

We generated a transgenic (Tg) mouse strain that over-expressed the mtDNA binding protein, TFAM, combined with EGFP (C57BL/6J-Tg(CAG-*Tfam*/EGFP)<sup>38Rin</sup>, *Tfam*/EGFP-Tg) (Fig. S1a). Compared with wild-type mice, the *Tfam*/EGFP-Tg mice displayed 9.10-fold (heart) and 5.67-fold (kidney) levels of total *Tfam* mRNA, but the same levels of endogenous *Tfam* (Fig. S1b). The TFAM/EGFP protein expression level was measured by Western blot using an antibody specific to GFP. The left ventricle, kidney and pancreas had a relatively higher expression of TFAM/EGFP, but the spleen and stomach had low levels. In Western blot analysis using cell fractionation of the heart and kidney, EGFP was detected in the mitochondrial fraction (Fig. S1c), and it was also found to be localized to the mitochondrial matrix using immunoelectron microscopy in the *Tfam*/EGFP-Tg mice (Fig. S1d). A gel shift assay showed a band shift of a linear DNA fragment with an mtDNA sequence dependent on the concentration of TFAM and TFAM/EGFP proteins, indicating that the TFAM/EGFP protein had DNA binding capacity (Fig. S1e).

We assayed the amount of mtDNA in 17 tissues from the *Tfam*/EGFP-Tg and wild-type mice using quantitative real-time PCR. Compared with their wild-type littermates, most tissues of the *Tfam*/EGFP-Tg mice had increased mtDNA copy numbers: cerebral cortex (2.51- and 1.61-fold in female and male, respectively), cerebellum (1.59- and 1.34-fold), heart (2.32- and 2.03-fold), thymus (1.52- and 2.67-fold), lung (4.28- and 2.43-fold), liver (0.92- and 1.03-fold), kidney (1.11- and 1.32-fold), adrenal body (1.45- and 1.69-fold), spleen (2.02- and 0.69-fold), pancreas (1.89- and 1.45-fold), stomach (2.14- and 1.89-fold), small intestine (1.87- and 1.60-fold), large intestine (2.09- and 1.06-fold), extensor digitorum longus (EDL) (2.83- and 1.90-fold), soleus (1.48- and 1.82-fold), ovary (1.59-fold), testis (1.10-fold) and tail (2.65- and 2.19-fold) (Fig. 1a). There was no significant difference between the amount of mtDNA in the tails of the wild-type mice and those of mtGFP-Tg mice that expressed mitochondrial-targeted EGFP (data not shown) [24], suggesting that the increase in mtDNA in the *Tfam*/EGFP-Tg mice was due to the effect of the *Tfam* over-expression.

### **Analysis of mitochondrial disease symptoms in *Tfam*/EGFP over-expressing mito-mice**

To investigate whether over-expression of *Tfam* improves the mitochondrial disease phenotypes, *Tfam*/EGFP over-expressing mito-mice (Tg-mito-mice) were generated by *in vitro* fertilization. Then the amounts of mtDNA in tail were estimated and we found that the Tg-mito-mice possessed 1.74 times as many as the average copy number of mtDNAs in their littermate mito-mice (Fig. 1b).

Among several littermates of Tg-mito-mice and mito-mice, the mice with more than 50%  $\Delta$ mtDNA were examined for their survival. Compared with mito-mice, the life spans of the Tg-mito-mice were significantly extended ( $P=0.01$  in Mantel-Cox test), and the median survival periods were 54% prolonged, from 247 days in the mito-mice to 381 days in the Tg-mito-mice (Fig. 2a).

Because most mito-mice show a high concentration of blood urea nitrogen (BUN) (reflecting renal dysfunction) and ultimately die due to renal failure [7], we measured the level of BUN in the mito-mice and Tg-mito-mice at eight months of age (Fig. 2b). An increased concentration of BUN was observed only in the mito-mice with more than 65%  $\Delta$ mtDNA in the tail, which was consistent with previous reports [7,23]. In the Tg-mito-mice group, the increase in BUN concentration was remarkably suppressed. However, there was one exception. A mito-mouse with 75.5%  $\Delta$ mtDNA had a normal BUN concentration, and it had a 1.58 times as much mtDNA copy number as the average of the control mito-mice at four weeks of age. These results indicate that the increased mtDNA copy number resulted in the recovery of kidney function. The kidneys of mito-mice with more than 80%  $\Delta$ mtDNA showed ischemia and increased size with a granulated surface [7]. In contrast, the Tg-mito-mouse kidney with 89%  $\Delta$ mtDNA, which contained 2.99 times as many mtDNA copies compared to the mito-mouse control kidney, showed a mild enlargement with a normal surface (Fig. 2c; left panel). A histological analysis showed dilatation of the cortical proximal and distal tubules in the renal tissues of the mito-mouse, which was recovered in the Tg-mito-mouse (Fig. 2c; right panels).

### **Histochemical analyses of cytochrome *c* oxidase**

Histochemical staining was used to evaluate the COX activity in the kidney (Fig. 3a) and heart (Fig. 3b). The kidneys of mito-mice with 83%  $\Delta$ mtDNA showed



dilation in the cortical proximal and distal tubules and a reduction of COX activity with a compensatory up-regulation of SDH activity [25]. The kidneys of the Tg-mito-mice with 89%  $\Delta$ mtDNA, however, had recovered both the COX activity and the histological characteristics of these tissues.

To confirm the effect of TFAM over-expression on the recovery of the mitochondrial activity in a single cell, cardiac muscle samples from the mito-mice and Tg-mito-mice (78% and 77%, respectively,  $\Delta$ mtDNA in the heart tissue block) were subjected to histochemical and quantitative single-fiber PCR analyses. Serial sections were used to compare the COX activity and proportion of  $\Delta$ mtDNA in a single muscle fiber. Consistent with a previous study [7], we found that there were fibers with variable proportions of  $\Delta$ mtDNA in the mito-mouse heart and that COX-positive fibers exhibited less than 85%  $\Delta$ mtDNA. In contrast, there were COX-positive fibers with more than 85%  $\Delta$ mtDNA in the Tg-mito-mouse heart (Fig. 3c). Although it is difficult to estimate the amount of mtDNA in single fibers because there is no effective internal control, the elevation of the threshold percentage of the  $\Delta$ mtDNA for maintaining COX activity was likely caused by the increase of mtDNA (2.27-fold higher than the mito-mouse control) due to the over-expression of TFAM.

### **The expression of mitochondrial tRNA was increased in Tg-mito-mice**

In cells with  $\Delta$ mtDNA, the amounts of mtDNA-coded proteins concurrently decreased regardless if the gene position was in the deleted region. The decrease in mtDNA-coded proteins should be due to the shortage of tRNAs[9], and compensation for this shortage was thought to be responsible for prolongation of life span (Fig. 2a), improved kidney function (Fig. 2b, c) and COX activity (Fig. 3a, b) in Tg-mito-mice. Therefore, we examined the amount of tRNA<sup>Ser(AGY)</sup> that coded in the deleted region.

Compared with mito-mouse tissues, the amounts of tRNA<sup>Ser(AGY)</sup> were increased as follows: 1.56-fold in the heart of the Tg-mito-mouse (78%  $\Delta$ mtDNA), which contained 2.27 times as many mtDNA copies as that of the mito-mouse (77%); 1.52-fold in the kidney of the Tg-mito-mouse (89%) which contained 2.99 times as many mtDNAs as that of the mito-mouse (83%); 2.42-fold in the soleus of the Tg-mito-mouse (70%) which contained 2.01 times as many mtDNAs as that of the mito-mouse (89%) (Fig. 4). The comparable result was observed using an additional set of mice (Fig. S2). These results showed that the amount of tRNA was moderately restored in the Tg-mito-mouse,

although it did not completely recover.

## Discussion

In this study we showed that *Tfam* over-expression improved the symptoms caused by mitochondrial deficiency with large deleted mtDNA. To elucidate the mechanism of this improvement, we created a new model for the pathogenicity of mutant mtDNA. The accumulation of  $\Delta$ mtDNA causes the simultaneous decrease of wild-type mtDNA, and a reduction in the tRNA supply. Consequently, the total protein levels of the respiratory complexes decrease as previously reported using cybrids with various proportions of  $\Delta$ mtDNA [7]. A certain level of respiratory complexes is required to maintain normal activity, so a loss of mitochondrial function occurs when the wild-type mtDNA decreases below the threshold. When the copy number of the wild-type mtDNA increases under the sufficient condition of gene expression, the tRNA supply and protein synthesis should increase, resulting in the improvement of mitochondrial function.

It has been recognized commonly that the proportion of pathogenic mutant mtDNA is responsible for the occurrence of mitochondrial dysfunction. However, our model proposes that the amount of wild-type mtDNA is important to maintain the mitochondrial function irrespective of the proportion of mutant mtDNA. There was a strong correlation between the amount of wild-type mtDNA and the life spans of both mito-mice and Tg-mito-mice (Fig. S3), indicating that the amount of wild-type mtDNA directly affects the life span of these mice.

However, there is a limitation for this therapeutic treatment. In mito-mice,  $\Delta$ mtDNA increases age-dependently [20], and mice born with a lower proportion of  $\Delta$ mtDNA may develop the mitochondrial diseases within their life span. In Tg-mito-mice, although the threshold percentage was elevated (Fig. 3c) by the increase in mtDNA levels, the mitochondrial dysfunction occurred when the proportion of  $\Delta$ mtDNA exceeded the threshold level. Thus, the mice could not be completely recovered even though the occurrence of the disease phenotype was extensively delayed and the life span was elongated (Fig. 2a).

Considering the model further, the mtDNA up-regulating factor does not need to be applied with a transgenic technique in the zygote. Viral vectors, gene guns or other undiscovered medicines might instead be used to increase the copy number of mtDNA. Furthermore, the method of increasing mtDNA copy number would be used to improve

mitochondrial dysfunction in adults. There are several reports in which the copy number of mtDNA has been increased using a non-transgenic approach. For instance, rats fed a high-fat diet and injected with heparin had increased levels of plasma free fatty acids and increased respiratory capacity, which is accompanied by an increase in the mtDNA copy number in skeletal muscle [26]. Raising the cytosolic  $\text{Ca}^{2+}$  with caffeine results in the increased expression of the peroxisome proliferative-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) gene [27], which increases mitochondrial biogenesis by activating the expression of *Tfam* and the subunits of the respiratory chain. It has also been reported that several cytokines, such as IL-1 $\beta$  and combinations of TNF $\alpha$  and IL-1 $\alpha$ , stimulate PGC-1 $\alpha$  activity through the activation of p38 MAPK, which is accompanied by increased mitochondrial gene expression and cellular respiration [28]. Pursuing the mechanisms underlying these phenomena may provide clues for the discovery of potent medicines for the treatment of mitochondrial diseases.

This is the first study to find that increase in the amount of total mtDNA rescues the impairment caused by pathogenic mtDNA, which is the cause of mitochondrial diseases. The therapeutic treatment in this study may be applied to human mitochondrial diseases, such as CPEO and KSS, especially in cases where the causal mtDNA mutation is the common deletion in which the deleted region extends from the *mt-Atp8* to the *mt-Nd5* gene [29], which is the same as the  $\Delta$ mtDNA in mito-mice.

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*Conflict of interest statement.* None declared.

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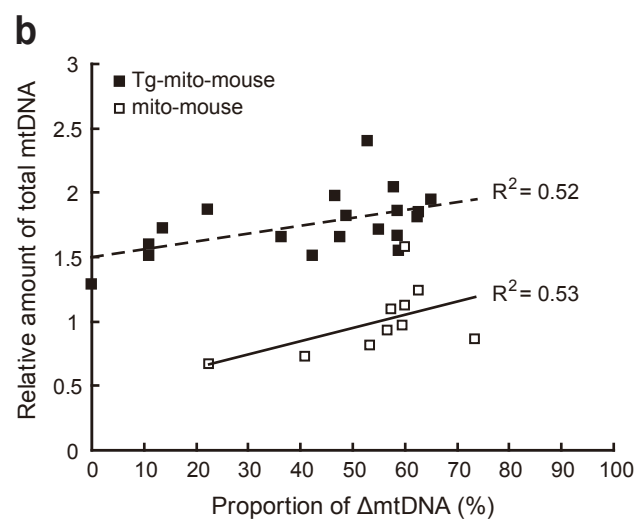
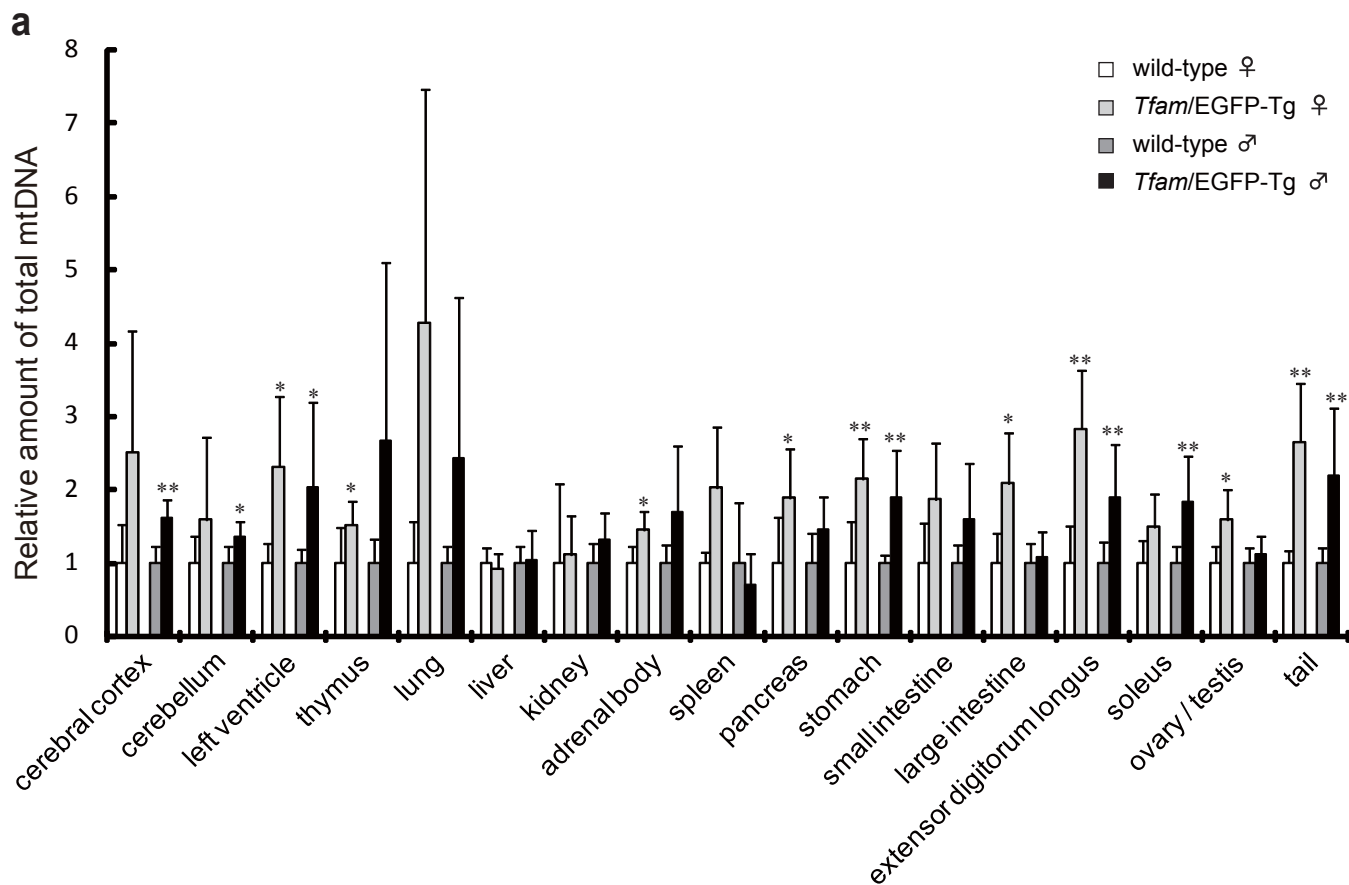


Figure 1. The effect of *Tfam*/EGFP over-expression on the amount of mtDNA. (a) The amount of mtDNA in tissues of wild-type and *Tfam*/EGFP-Tg mice. The vertical scales indicate the relative amounts of mtDNA compared with the mean of the wild-type samples in each sex. The white bar, lighter dark bar, dark bar and black bar show wild-type females ( $n=10$ ), Tg females ( $n=5$ ), wild-type males ( $n=7$ ) and Tg males ( $n=8$ ), respectively.  $*P < 0.05$  and  $**P < 0.01$ . (b) The amounts of total mtDNA in the tail of mito-mice and Tg-mito-mice were measured by the comparative real-time PCR method. On average, the Tg-mito-mice contained 1.74 times as many mtDNA copies as the mito-mice in the four-week-old tail. Both mouse strains had positive correlations between the proportion of  $\Delta$ mtDNA and mtDNA copy number. The solid and dashed lines indicate the regression lines for the mito-mice and Tg-mito-mice, respectively.

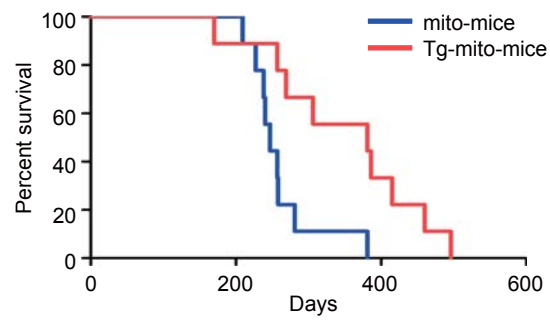
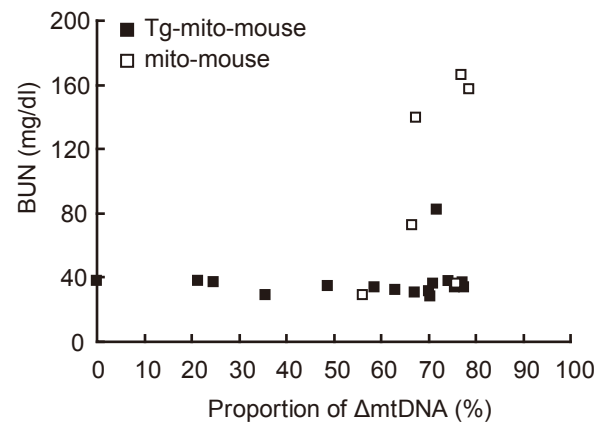
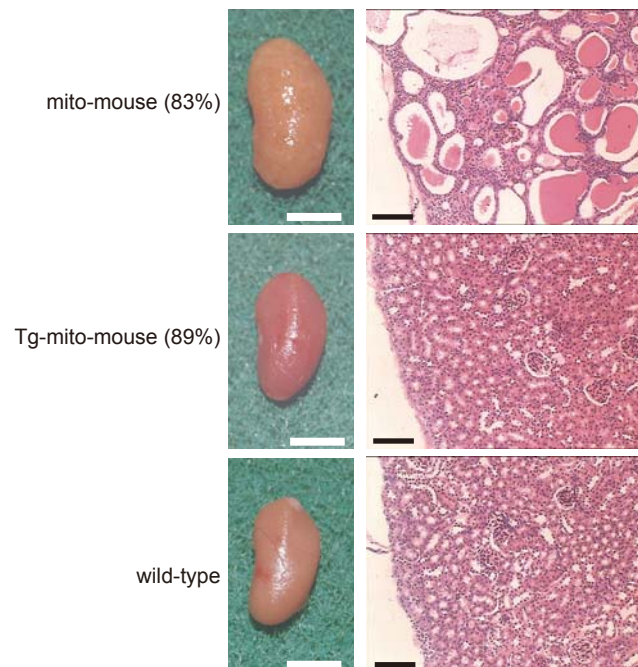
**a****b****c**

Figure 2. The effects of *Tfam*/EGFP over-expression on the disease phenotypes of mito-mice. (a) Kaplan-Meier survival curves of mito-mice ( $n=9$ ; blue line) and Tg-mito-mice ( $n=9$ ; red line) with more than 50%  $\Delta$ mtDNA. (b) An association between the proportion of  $\Delta$ mtDNA and blood urea nitrogen (BUN) in eight-month-old mice. The black squares indicate Tg-mito-mice and the white squares indicate mito-mice. (c; left panels) The morphology of the kidneys from a mito-mouse, a Tg-mito-mouse and a wild-type mouse at eight months of age. The Tg-mito-mouse contained 2.99 times as many mtDNA copies in the kidney as the mito-mouse. Bar, 5 mm. (c; right panels) The kidney sections were stained with hematoxylin and eosin to observe the histopathology of the renal cortex. Bar, 0.1 mm.

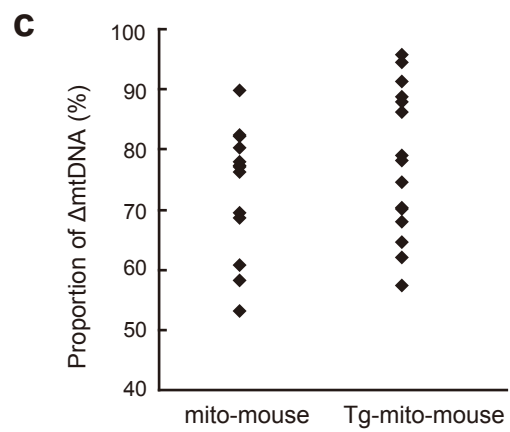
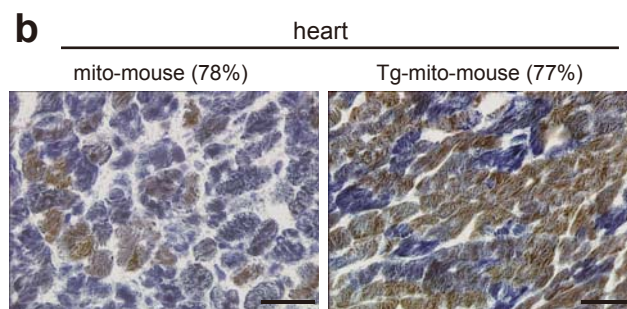
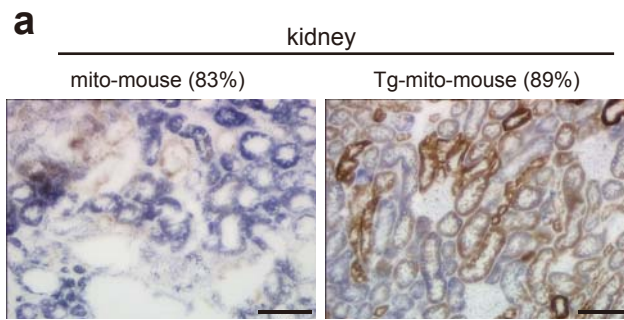


Figure 3. The tissue distribution of COX-positive and COX-negative cells in mito-mice and Tg-mito-mice. (a, b) The tissue sections were sequentially stained for the activity of the COX (brown) and SDH (blue) of the electron transport chain. The brown staining depicts cells with intact COX activity. The blue-stained cells have lost their COX activity but have retained SDH activity, which is only encoded by nuclear genes. The mito-mouse and the Tg-mito-mouse displayed 83% and 89%  $\Delta$ mtDNA in the kidney, respectively (a), and 78% and 77% in the heart, respectively (b). The Tg-mito-mouse displayed 2.99 and 2.27 times as many mtDNA copies as the mito-mouse in the kidney and heart, respectively. Bar, 100  $\mu$ m in kidney and 50  $\mu$ m in heart. (c) The proportion of  $\Delta$ mtDNA in a COX-positive single fiber of heart muscle.

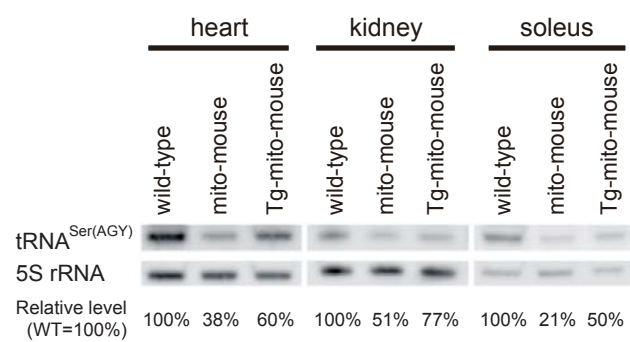




Figure 4. The amount of mitochondrial tRNA<sup>Ser(AGY)</sup> in heart, kidney and soleus measured by Northern blot analysis. Relative amounts of tRNA<sup>Ser(AGY)</sup> compared with wild-type tissues are indicated. The proportions of  $\Delta$ mtDNA in each mouse were as follows: heart, 78% in Tg-mito-mouse and 77% in mito-mouse; kidney, 89% in Tg-mito-mouse and 82% in mito-mouse; and soleus, 70% in Tg-mito-mouse and 89% in mito-mouse.

## Supplementary Materials

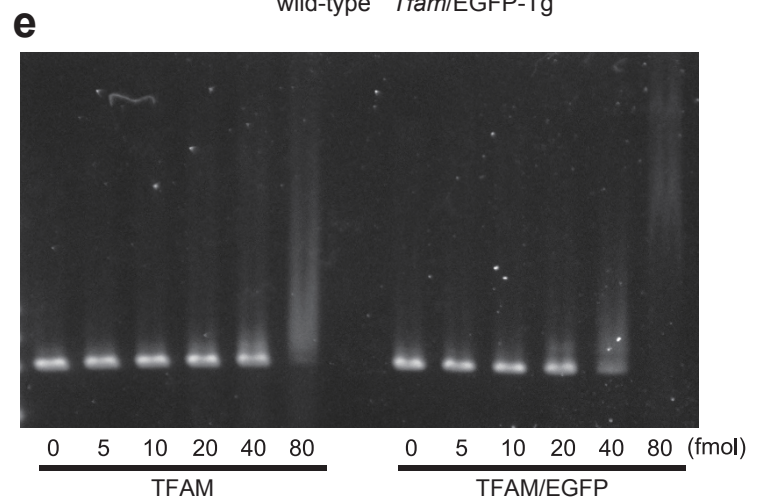
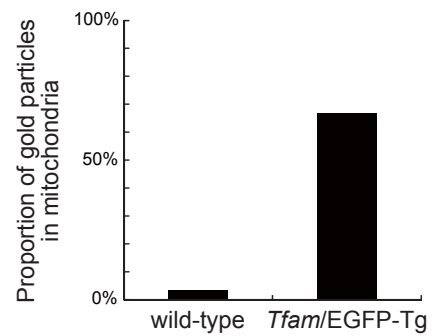
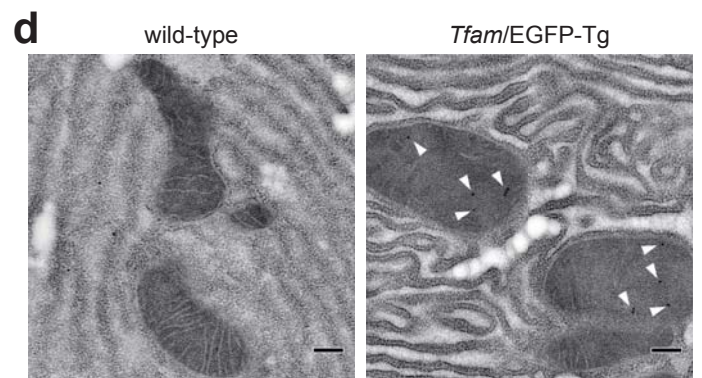
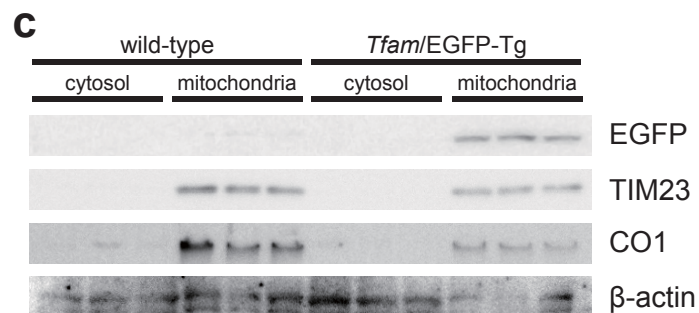
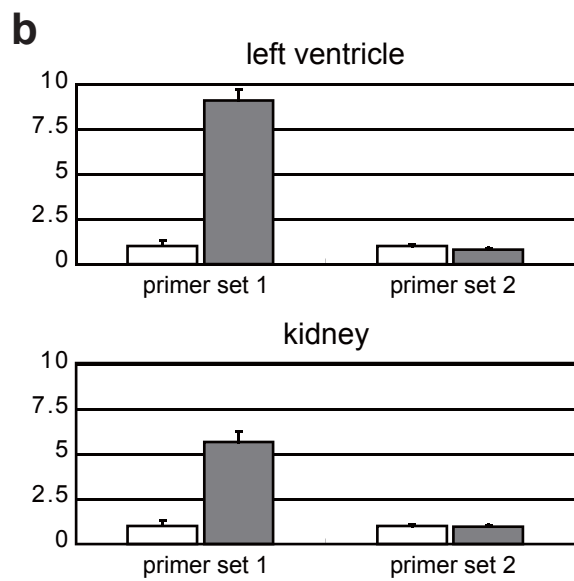
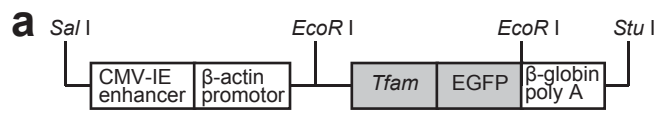
### Figure Legends

Figure S1. Generation and characterization of *Tfam*/EGFP-Tg mice strain. (a) Schematic structure of the transgene. Mouse TFAM and EGFP cDNA were ligated and then introduced into a pCAGGS expression vector that contained the cytomegalovirus enhancer, the chicken  $\beta$ -actin promoter and the rabbit  $\beta$ -globin poly-A. The DNA fragment digested with *Sal*I and *Stu*I was isolated for microinjection. (b) Real-time RT-PCR analysis of the expression of *Tfam* in wild-type (white bar,  $n=3$ ) and *Tfam*/EGFP-Tg (gray bar,  $n=3$ ) mice. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and 18S rRNA genes were used as the control in the Northern blot and the RT-PCR, respectively. (c) Western blot analysis of cell fraction. The mitochondrial fraction was determined by anti-TIM23 and anti-CO1 antibodies. The TFAM/EGFP, which was detected with the anti-GFP antibody, was observed only in the mitochondrial fraction of the *Tfam*/EGFP-Tg mice. (d) Immunoelectron micrographs of pancreases from *Tfam*/EGFP-Tg and wild-type mice. The gold label for TFAM/EGFP was present in the mitochondria of the pancreas from the *Tfam*/EGFP-Tg mouse (white arrowhead), but not in that of the wild-type mouse. Bar, 200 nm. The rates of gold particles in the inner mitochondria were 66.7% (114/171 in Field of View) in the Tg mice and 3.3% (1/30 in FOV) in the wild-type mice (bar chart). (e) Gel shift assay to assess DNA binding capacity of TFAM and TFAM/EGFP. An 868-bp fragment of mtDNA (20 fmol) was incubated with increasing amounts of TFAM and TFAM/EGFP proteins.

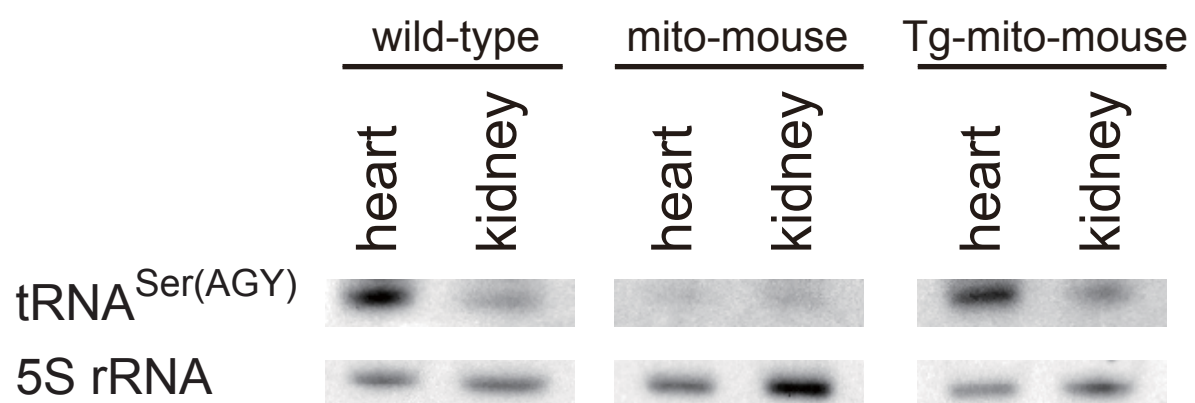
Figure S2. The effect of *Tfam* over-expression on the mitochondrial transcription. The amounts of mitochondrial tRNA<sup>Ser(AGY)</sup> in heart and kidney were estimated by the Northern blot analysis. Compared to mito-mice (55.7%  $\Delta$ mtDNA in 4-weeks tail), the Tg-mito-mouse (63.9%) possessed 1.21-fold (heart), 3.13-fold (kidney) higher in the levels of tRNA<sup>Ser(AGY)</sup>.

Figure S3. The life span plotted with the amount of wild-type mtDNA. The horizontal scale shows the relative copy number compared with the average amount of

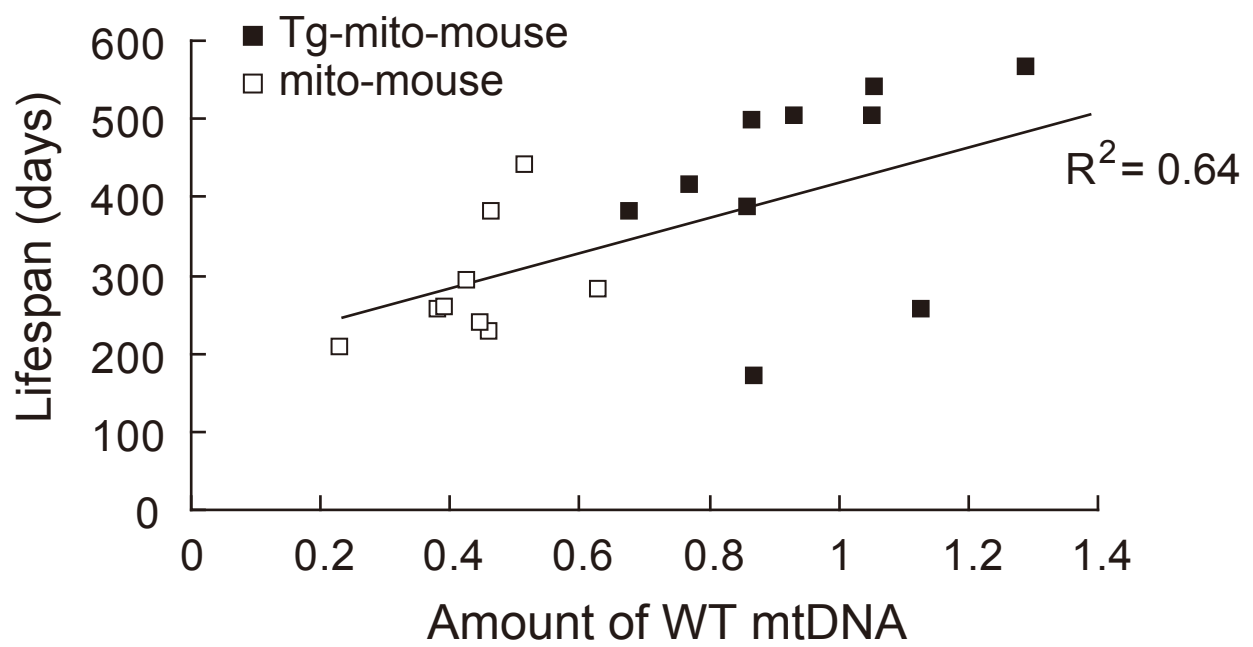
mtDNA of the mito-mice.



**Figure S1**



**Figure S2**



**Figure S3**

## **Materials and Methods**

### **Construction of the expression vector**

The signal sequence of mouse *Tfam* (GenBank Accession No. BC001987) was synthesized by PCR with primers 5'-GGC GAA TTC CTC GAG GCC ACC ATG GCG CTG TTC CGG GGA ATG T-3' and 5'-CAT ACG CGT ATG CTC AGA GAT GTC TCC GGA TCG T-3' using RT-PCR fragments derived from C57BL/6J (B6) as a template. The coding region of EGFP was amplified by PCR with primers 5'-CAT ACG CGT ATG CTC AGA GAT GTC TCC GGA TCG T-3' and 5'-CAT ACG CGT ATG GTG AGC AAG GGC GAG GA-3' using the recombinant plasmid pEGFP-Tub (Clontech) as a template. Both PCR products were digested with *EcoRI* and *MluI*, and the ligated pCAGGS expression vector [1] was digested with *EcoRI*.

### **Generation of transgenic mice**

DNA fragments for transgenesis were removed by double digestion with *SalI* and *StuI* (Fig. 1a). These fragments were separated by agarose gel electrophoresis from the cloning vector and then purified with a MonoFas DNA purification kit I (GL Science). The purified DNA fragments were injected into the pronuclei of the fertilized mouse (B6) oocytes according to the standard procedure [2]. To identify the transgenic mice, genomic DNA were prepared from the tails using a Gentra Puregene Tissue kit (QIAGEN) and two sets of primer pairs, (A) 5'-GCT CTA GAG CCT CTG CTA ACC-3', 5'-TGA ACA GCT CCT CGC CCT TGC TC-3' and (B) 5'-TGA GCA AAG ACC CCA ACG AGA AGC-3', 5'-TTA GCC AGA AGT CAG ATG CTC AAG-3', were synthesized to amplify the PCR fragments (258 and 248 bp) specific to the EGFP sequence [3].

### **Preparation of total RNA from mice tissues**

Tails (about 5-mm lengths), hearts, kidneys and skeletal muscle samples for RNA preparation were cut and immediately frozen in liquid nitrogen. The frozen samples were crushed and homogenized (SK-100, Tokken). Then, the total RNA was isolated using the ISOGEN (NIPPON GENE) or QIAeasy plus (QIAGEN) kits.

### **Real-time RT-PCR analysis of *Tfam* mRNA**

A One Step SYBR RT-PCR Kit (TaKaRa) and the following primer sets were used for real-time RT-PCR; *Tfam*: TaKaRa MA027412 (primer set 1), TaKaRa MA076007 (primer set 2) (the set was designed in the 3' UTR region of the *Tfam* sequence), *Rps18*: TaKaRa 050364.

### **Preparation of the mitochondrial fraction**

Sixty milligrams of fresh kidneys were used to prepare the cytosol and mitochondrial fraction. The samples were homogenized with Tissue-Ruptor (QIAGEN) for 10 seconds followed by a Qproteome Mitochondria Isolation Kit (QIAGEN) treatment.

### **Western blot analysis**

Proteins from the tissue samples were separated by SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare). After being blocked with 5% BSA in TBS for  $\beta$ -Actin or 0.5% skim milk for others, the membranes were incubated with a specific antibody (anti-GFP: MBL, anti-CO1 and anti- $\beta$ -Actin: Abcam, anti-TIM23: BD). Horseradish peroxidase-conjugated goat antibody to rabbit IgG (Bio-Rad) or mouse IgG (Abcam) was used as a secondary antibody. Immun-Star HRP Substrate (Bio-Rad) was used for the detection.

### **Immunogold electron microscopy**

The pancreas and EDL of *Tfam*/EGFP-Tg or wild-type mice were fixed with a solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 60 min at 4°C. The tissues were washed with PBS five times, dehydrated with a graded ethanol series, embedded in LR White and finally polymerized with UV light at -20°C, as reported previously [4]. Ultra-thin sections of LR White-embedded samples were prepared and mounted on nickel grids. After being blocked with 0.5% BSA in PBS for 15 min, the sections were incubated with a rabbit anti-GFP antibody (1:1,000; MBL, Japan) overnight at 4°C. The sections were washed with PBS and incubated with a goat anti-rabbit IgG coupled to 15-nm colloidal gold (1:50; BioCell Research Laboratories, Cardiff, UK) for 60 min at room temperature. For the control experiments, non-immune normal rabbit IgG and the immunogold were used. After being washed with PBS and distilled water, all sections



were stained with uranyl acetate and lead citrate and then examined in a JEM 1230 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

### **DNA Binding Assay**

DNA fragments that encode TFAM and TFAM/EGFP with a 6×His-tag at the C-terminus were generated and cloned into the vector pCAGGS. The recombinant proteins were expressed in COS7 cells and purified using the Ni-NTA Spin Kit (QIAGEN).

The 868 bp fragment of mtDNA was amplified using primer pairs 5'- AAA AAG ATA AAA GGA ACT CGG CAA ACA AGA-3' and 5'- ATA AAG AAC ACT ATT AGG GAG AGG ATT TGA-3'. The fragment (20 fmol) was incubated with increasing concentrations (5-80 pmol) of TFAM or TFAM/EGFP proteins in a binding buffer (150 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 10 mM Tris, pH 7.4), which was contained in the Electrophoretic Mobility Shift Assay Kit (Molecular Probes). The reaction mixtures were incubated for 20 min at room temperature and subsequently analyzed by electrophoresis in a 1% agarose gel with 1xTBE. The fragment of mtDNA in the gel was detected using a SYBR Green EMSA nucleic acid gel stain.

## References

- [1] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, *Gene* 108 (1991) 193-199.
- [2] B. Hogan, *Manipulating the mouse embryo : a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1994.
- [3] H. Shitara, A. Sato, J. Hayashi, N. Mizushima, H. Yonekawa, C. Taya, Simple method of zygosity identification in transgenic mice by real-time quantitative PCR, *Transgenic Res* 13 (2004) 191-194.
- [4] H. Suzuki, R.L. Kinlough-Rathbone, M.A. Packham, J.F. Mustard, K. Tanoue, H. Yamazaki, Localization of fibrinogen during ADP- or thrombin-induced aggregation of washed rabbit platelets, *J Histochem Cytochem* 38 (1990) 869-874.