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Neurological phenotype and reduced lifespan in heterozygous *Tim23* knockout mice, the first mouse model of defective mitochondrial import

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ABSTRACT

The Tim23 protein is the key component of the mitochondrial import machinery. It locates to the inner mitochondrial membrane and its own import is dependent on the DDP1/TIM13 complex. Mutations in human DDP1 cause the Mohr-Tranebjaerg syndrome (MTS/DFN-1; OMIM #304700), which is one of the two known human diseases of the mitochondrial protein import machinery. We created a *Tim23* knockout mouse from a gene trap embryonic stem cell clone. Homozygous *Tim23* mice were not viable. Heterozygous F1 mutants showed a 50% reduction of Tim23 protein in Western blot, *a neurological phenotype* and a markedly reduced life span. Haploinsufficiency of the *Tim23* mutation underlines the critical role of the mitochondrial import machinery for maintaining mitochondrial function.

1. Introduction

In mouse and man, the small mitochondrial genome codes for only 13 of about 1500 mitochondrial proteins [1]. The vast majority of mitochondrially located proteins is encoded by nuclear genes and then imported into the organelle by a complex import machinery [2-4]. The TOM (translocase of outer mitochondrial membrane) complex is the gate for almost all imported mitochondrial precursor proteins [5]. The TIM23 (translocase of inner mitochondrial membrane) complex enables further import of precursors into the mitochondrial matrix or inner membrane. The TIM22 complex is responsible for the insertion of multispanning proteins harbouring internal target information into the inner membrane of mitochondria, assisted by the soluble TIM9/10 and TIM8/13 protein complexes [4]. The Tim23 protein (Tim23p) is a key component of the mitochondrial import machinery in all mitochondria-bearing eukaryotes [6] as it is necessary for the import of at least all matrix proteins [7]. In *S. cerevisiae* and *Neurospora crassa*, *Tim23* is essential [8]. Tim23p is the channel forming part of the TIM23 complex located in the inner mitochondrial membrane [9]. In yeast, the TIM23 complex comprises Tim23p and the integral membrane components Tim14p, Tim17p and Tim50p. In addition, Tim44p and Tim16p are peripheral membrane proteins associated to the complex [10]. Mitochondrial Hsp70p is recruited to the TIM23 complex via Tim44p as part of the import motor to bind incoming precursors [11].

Two human diseases have been described that are caused by defects of the mitochondrial protein import machinery: the Mohr-Tranebjaerg syndrome and the syndrome of dilated cardiomyopathy with ataxia (DCMA; OMIM #610198) [12]. DCMA is a type of 3-methylglutaconic aciduria (type V), which is clinically characterized by severe, early onset dilated cardiomyopathy, growth retardation, ataxia and optic atrophy. It is caused by a splice mutation in the *DNAJC19* gene, which is supposed to be the human homolog of yeast *Tim14* [13].

The Mohr-Tranebjaerg syndrome (MTS; OMIM #304700; also called deafness dystonia syndrome, DDS) shows X-linked recessive inheritance and is characterized by progressive sensorineural hearing loss, dystonia, mental deficiency, and visual disability [14,15]. It is caused by a mutation of the deafness/dystonia protein1 gene (DDP1), also called translocase of the inner membrane 8a (TIM8A). Ddp1p is part of a hetero-oligomeric complex in the intermembrane space of mitochondria, which contains Tim13p in addition to Ddp1p [16]. In yeast, this complex is involved in the import of some mitochondrial

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Fig. 1. Structure of the *Tim23* gene in mouse and the site of integration of the gene trap vector. Exons are shown as thick bars, introns as small connections.

precursor proteins via the TOM22 complex, especially that of Tim23p [17,18]. The Tim8p/Tim13p complex is not essential in yeast but lack of this complex leads to a temperature-sensitive growth defect. In vitro, import of Tim23p into mitochondria from Δ*Tim8*/Δ*Tim13* yeast cells is impaired at low membrane potential [18]. The involvement of Ddp1p in Tim23p import has also been shown in human cells. As additional substrates for Ddp1p-dependent import, citrin and aralar1 were identified in these cells [19]. Immunostaining indicates higher expression of the DDP1/TIM13 complex in some neuronal cells, for example cerebella Purkinje cells, and lower expression in heart and muscle tissue [20].

Tim23p was the first substrate of the DDP1/TIM13 complex identified. Because of its essential function it was hypothesized that decreased levels of Tim23p in the inner mitochondrial membrane could be the cause of MTS [19,21]. Here we established a *Tim23* mutant mouse using a genetrap clone. Homozygous *Tim23* died before implantation, while heterozygous *Tim23* mice had a significantly reduced lifespan with incomplete penetrance in the F1 generation. A number of heterozygous *Tim23* mice displayed signs of early aging, including alopecia and kyphosis.

2. Materials and methods

2.1. Establishment of a Tim23 mutant

Clone W023C08 was produced within the German Gene Trap Consortium (GGTC) [22] and matched to *Tim23* exon 6 according to the 5' RACE sequence. Mutant mice were generated after blastocyst injection and resulting chimeras were bred to C57BL/6 females. F1 animals were intercrossed in order to generate homozygous mutants.

2.2. Blastocyst genotyping

Blastocysts were harvested by uterine flushing 3.5 days after a vaginal plug was observed and collected individually in 30 mM HEPES-buffer, pH 7.2. The blastocysts were heated to 95 °C for 10 min and used directly as template for the genotyping PCR reactions.

2.3. β-galactosidase staining of mouse embryos

Isolated mouse embryos were fixed on ice for 20–45 min in 2% paraformaldehyde and 0.1% glutaraldehyde. After rinsing the sample three times with PBS β-galactosidase activity was monitored overnight as described elsewhere [23].

2.4. Biochemical procedures

Mitochondria enriched fractions were isolated from different mouse tissues by differential centrifugation as described elsewhere [24]. Blotting to polyvinylidene difluoride or nitrocellulose membranes and immunodecoration was done according to standard procedures and visualization was done by the ECL method (Amersham Biosciences).

The steady state rates of oxygen consumption of digitonin permeabilized embryonic stem cells was recorded as a decrease in oxygen concentration over time by using the two-channel high resolution respirometer (Oroboros Oxygraph; Paar KG, Graz, Austria) according to the manufacturer instructions. The rate of mitochondrial respiration was assessed in response to the consecutive addition of glutamate/malate, ADP, rotenone and succinate to determine the apparent kinetic parameters of regulation and respiratory control ratio.

Isolated respiratory chain complex IV activity and complex II + III activity were measured on isolated mitochondria according to [25].

2.5. Phenotypic characterization of the mice

2.5.1. SHIRPA

A modified SHIRPA (**S**mithkline Beecham, MRC **H**arwell, **I**mperial College, the **R**oyal London hospital **p**henotype **a**ssessment) protocol was performed as described [26].

2.5.2. Grip strength

The muscle forelimb strength of the mice was measured using a grip strength meter (TSE; Bad Homburg, Germany). The animals grasped a horizontal metal bar while being pulled by their tail. A sensor allowed measurements of up to 600 lb. Five trials within 1 min were performed for each mouse.

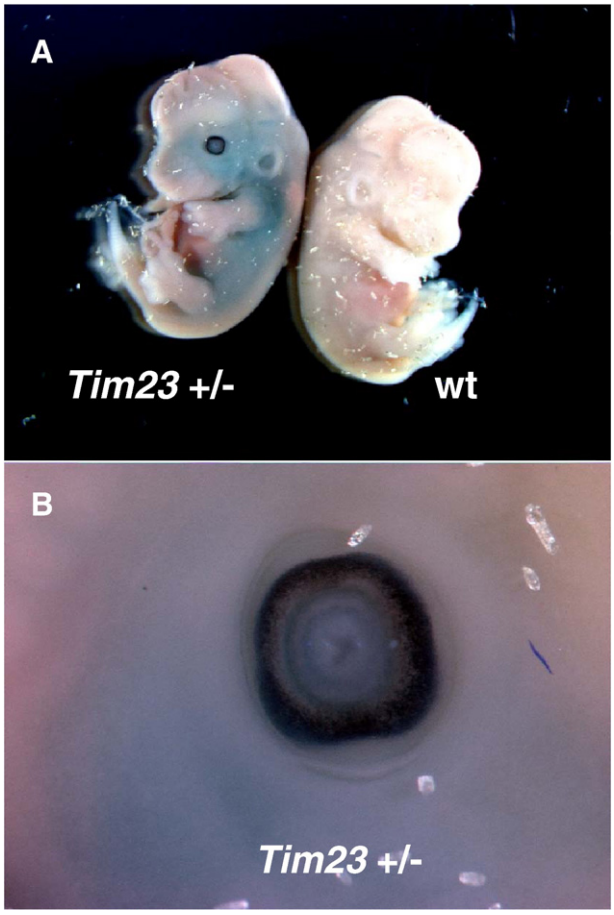

Fig. 2. LacZ histochemistry on whole embryos. The pictures show (A) whole embryos stained with lacZ and (B) the eye of one stained *Tim23*+/− embryo.

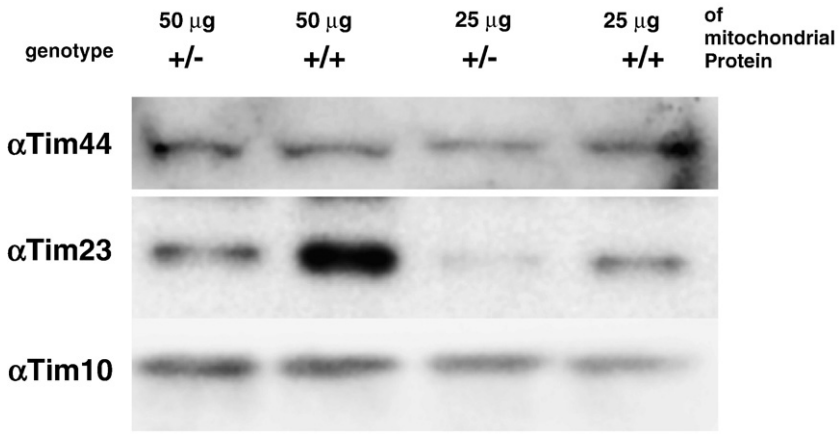

Fig. 3. Levels of TIM23 are reduced in *Tim23*+/− mice. 25 µg and 50 µg of mitochondrial protein were separated by SDS-PAGE, blotted and decorated with antibodies against TIM44, TIM23 and TIM10. The staining shows less content of TIM23 in *Tim23*+/− mitochondria in comparison to *Tim23*+/+ mitochondria (second row), while the levels of TIM44 and TIM10 are virtually unchanged (first and third row).

2.5.3. Rotarod

To measure motor coordination, balance and motor learning ability, a rotarod apparatus (TSE, Bad Homburg, Germany) was used [27-29]. The rotarod unit consists of a computer-controlled motor-driven rotating spindle and four lanes for four mice. Infrared beams are used to detect the falling of the mice. The mean latency to fall off the rotarod was recorded. Passive rotation behavior was noted. Mice that rotated passively for three times were scored as fallen.

The machine was set up in an environment with minimal stimuli such as noise and movement. On the first day, the mice were habituated to the device in two 180 second-sessions at constant speeds of 12 and 20 rpm. In the motor coordination performance test on the second day, mice exerted four trials with linear accelerating speed from 4 to 40 rpm within 300 s.

3. Results

3.1. Creation of a Tim23 mutant

The murine *Tim23* gene (NM_016897) is located on chromosome 14, contains seven exons and codes for a transcript of 1142 bp (UCSC-Genome Browser mouse built 36; Assembly Feb 2006: chr.14 31009177-31030880). In the selected gene-trap clone W023C08, the pT1ßgeo gene trap vector had inserted between exons 6 and 7 of the gene, as determined by 5' RACE and cDNA sequencing of trapped transcripts as well as genomic analysis (Fig. 1). Genomic sequencing revealed that the vector integrated in position chr.14:31010477 (mm9 assembly).

3.2. Tim23 is essential for embryonic development

After intercrosses of *Tim23*+/− animals, no homozygous *Tim23*−/− offspring was found at weaning age. We did not exclude an effect in haploid gametes of Tim23 heterozygotes, however reciprocal crosses of heterozygous *Tim23* with wild type showed, that the *Tim23* mutation is transmitted through both maternal and paternal germlines.

In order to determine the developmental stage of lethality, we first genotyped embryos 10 days post coitum (dpc). Among more than 50 embryos analyzed, we did not recover any homozygotes, indicating an earlier stage of lethality. Similarly, no *Tim23*−/− were recovered from 37 embryos at 3.5 dpc, indicating that homozygous *Tim23*−/− mutants die before implantation.

3.3. A LacZ fusion protein is expressed in Tim23+/− *mice*

To determine the expression pattern of *Tim23* in embryos, we performed lacZ histochemistry on whole embryos. In embryos at ages 10 and 14 dpc, the fusion protein with β-galactosidase activity was expressed ubiquitously indicated by an even staining of the whole embryos. Particular intense staining was found in the eye lens (Fig. 2). In the light that the GT vector had inserted in the last intron of *Tim23*, we expected the fusion protein to be recognized by a polyclonal antibody specific for Tim23p, which is directed against a C-terminal peptide of human Tim23p [30]. However, the fusion protein could not be detected by Western blotting and immunodecoration neither in mitochondria nor in cytosol (data not shown). Therefore we hypothesize that the fusion of Tim23 and β-galactosidase is instable or modified posttranslationally.

3.4. Reduced Tim23p levels in Tim23+/− *mutants*

In order to monitor the effect of the loss of one functional allele of *Tim23*, we analyzed the level of Tim23p. Mitochondria were isolated from liver of *Tim23*+/+ and *Tim23*+/− mice. The mitochondrial proteins were separated by SDS-PAGE and blotted on PVDF membrane. Immunodecoration with αTim23p antibodies showed a 50% reduction of Tim23p levels in mitochondria of *heterozygous* mice as compared to their wildtype littermates (Fig. 3). Next, we analysed the respiratory chain complex activities of embryonic stem cells from the gene-trap clone W023C08 and three controls including two clones with

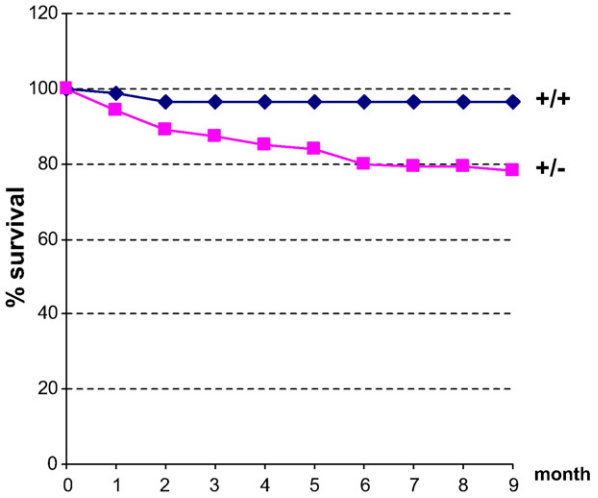

Fig. 4. Kaplan-Meier curve for *Tim23*+/+ and *Tim23*+/− mice. Of 198 born mice investigated (89 *Tim23*+/+, 109 *Tim23*+/−) 24 *Tim23*+/− mice died within the first 9 months, while only 3 *Tim23*+/+ died.

unrelated vector integrations. For the in situ analysis of mitochondrial respiration we used polarographic measurements by means of Clark-type oxygen electrodes and digitonin permeabilized cells. In addition we biochemically measured the respiratory chain complex II + III and IV activities of isolated mitochondria biochemically. By neither the method we could detect a significant difference of the heterozygous *Tim23* cells as compared to controls (data not shown).

3.5. Heterozygous Tim23+/− mice show a reduced lifespan and a neurological phenotype

Phenotypic characterization of heterozygous *Tim23*+/− mice included monitoring of lifespan, SHIRPA testing at age 16 weeks, examination of grip strength and a rotarod test.

Tim23+/− mice showed a significantly increased mortality rate in comparison to wildtype. Until the age of 9 month, 24 of the 109 *Tim23* +/− died but only 3 of the 89 *Tim23*+/+ mice ($p<0.005$, Fig. 4).

Grip strength measurements revealed a significantly reduced forelimb grip strength in female *Tim23*+/− mice ($n=6$). In males there was also a trend towards reduction of grip strength but this difference did not reach significance (Fig. 5A).

Rotarod analysis showed a significantly reduced dwell time on the rotating drum at higher speed for male *Tim23*+/− mice in comparison to wt mice ($n=3$). This indicates reduced ability of motor coordination and balance in male *Tim23*+/− (Fig. 5B). The effect could not be detected in female mice ($n=6$).

No apparent impairment of hearing and vision could be detected based on the visual placing behaviour in the SHIRPA analysis and on the reaction to a clickbox test.

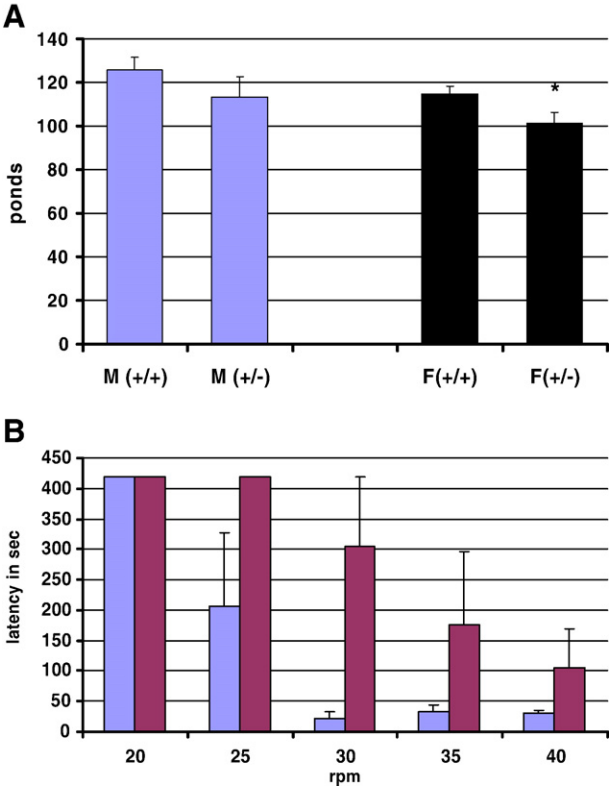

Fig. 5. Grip strength and rotarod measurements of Tim23+/+ and Tim23+/− mice. (A) Measurement of grip strength of 4 month old male individuals ($n=4$) and female individuals ($n=6$). Grip strength of female Tim23+/− mice is significantly reduced in comparison to female Tim23+/+. (B) Rotarod tests for 4 month old male individuals. Shown is the latency time on the drum with increasing speed at the second day of training (see Materials and methods). At higher drum speed the latency time of Tim23+/− mice ($n=3$, dark bars) is significantly shorter than the latency time of Tim23+/+ mice ($n=3$?, bright bars).

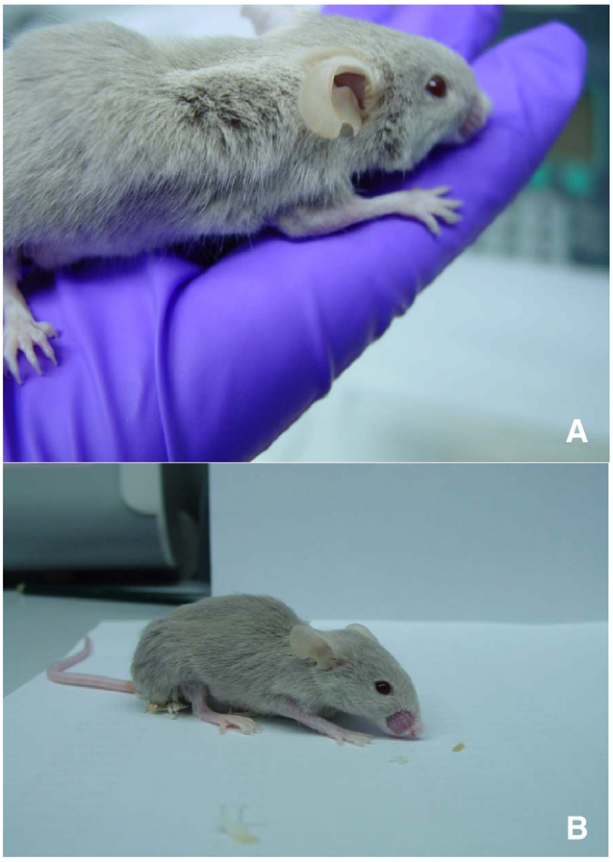

Fig. 6. Some *Tim23*+/− mice showed symptoms of early ageing. The pictures show an 8 month old individual with kyphosis (A) and alopecia (B).

3.6. Some F1 heterozygous Tim23+/− animals display signs of premature aging

Mitochondrial dysfunction is hypothesised to limit mammalian lifespan [31]. For example mice with a defect of mitochondrial DNA polymerase gamma (Polga) show several features of accelerated aging [32,33]. When analysing *Tim23*+/− heterozygote mice we found symptoms of premature ageing like alopecia and kyphosis in several heterozygous animals, not seen in wt controls (Fig. 6).

3.7. Backcross of Tim23+/− animals on C57BL/6J and 129SvP genetic backgrounds results in loss of the phenotype

Since incomplete penetrance of the ageing phenotype was obvious, the *Tim23* mutation was backcrossed on a C57BL/6J and 129SvP genetic background in order to increase the stability of the phenotype. Unexpectedly the phenotype becomes weaker from the N2 generation on. In the fifth generation no difference between *Tim23*+/+ mice and *Tim23*+/− mice was evident. To ascertain the reason for that, we investigated the steady state level of Tim23p in mice mitochondria isolated from brain, liver and heart tissues by Western blotting. In contrast to determination in F1 *Tim23*+/− mice, the *Tim23*+/− mice of generation 2 to 5 showed a level of Tim23p which was undistinguishable from that in *Tim23*+/+ mice (data not shown). However, the genotyping clearly showed the hererozygosity concerning *Tim23*.

4. Discussion

The unability to recover homozygous *Tim23*−/− mutants at any analyzed stage of development and the transmission of *Tim23*-alleles through egg and sperm cells are strong indications that *Tim23* is essential even before implantation. Considering that *Tim23* is essential

in *S. cerevisiae* and *N. crassa* and the non-redundant role of Tim23p in the biogenesis of mitochondria, our findings are in accordance with the hypothesis that *Tim23* is an essential gene in mouse as well.

The Tim23+/− mouse is the first mouse model of defective mitochondrial import. Until now, there is no human disease with Tim23 mutations but the mouse model indicates that heterozygosity for this gene defect is compatible with life and may manifest as a premature aging phenotype. While the human Mohr-Tranebjaerg syndrome is closely related from a molecular view (as Tim8a defects compromise Tim23 import), the Tim23+/− mouse does not recapitulate its typical features such as deafness, dystonia and visual disability [14]. This is not unexpected as there is often a marked phenotypical difference between man and mouse even if true homologs are knocked out. Instead, the Tim23+/− mouse showed a neurological phenotype of reduced grip strength in females and impaired motor coordination in males. The lack of these signs in the respective other gender is most probably a statistical effect due to the small number of animals tested.

The signs of early aging shown by some heterozygous *Tim23*+/− mice with reduced TIM23 levels may be explained by an overall reduction of the ratio of mitochondrial import of mitochondrial precursors via the Tim23 complex. This reduction may result in a general impairment of mitochondrial function. Patients with decreased Hsp60p levels in mitochondria are described as having an atypical mitochondrial disease with multisystemic failure [12]. If this is due to the severity of impairment of mitochondrial function and if less severe impairment can lead to MTS is not known.

Several mouse models are reported to show an aging phenotype with marked resemblance to human aging. Aging symptoms found in humans are for example weight loss, reduced fat storage, alopecia, osteoporosis, and loss of Complex IV (COX) activity in several tissues. Mitochondria are thought to play an important role in aging. Increasing numbers of mtDNA mutations and deletions can be found upon aging [34]. One prominent mouse model for aging, reported 2004, is the so called mtDNA mutator mouse, containing a mitochondrial DNA polymerase γ lacking its proof-reading activity [32]. The mouse is characterized by reduced lifespan, weight loss, reduced fat storage, alopecia, kyphosis, osteoporosis, heart enlargement, and loss of COX activity in several tissues. Impairment of oxidative phosphorylation can lead to increased production of reactive oxygen species (ROS), which can damage mitochondria and cells irreversibly [35-37]. This is integrated in the mitochondrial theory of aging, which implicates ROS, mtDNA damage, and progressive respiratory chain dysfunction as key factors, being mutually related in a vicious circle [38].

During the outcrossing experiments, heterozygous *Tim23*+/− mice lost the phenotype on both, the C57BL/6J and the 129SvP genetic backgrounds. Possible explanations for the disappearance of the phenotype is increased robustness due to the different genetic background and positive selection for mutants with relatively high residual *Tim23* expression, which may be more fertile than the ones with the lowest residual expression.

In the *Tim23*+/− mouse with reduced Tim23p levels, impaired mitochondrial import could lead to reduced respiration and increased oxidative stress. In Fig. 3 it is shown, that the steady state levels of the used markers Tim10p and Tim44p are not reduced. Tim10p is imported independently of Tim23p, but Tim44p needs Tim23p to be imported. If impaired mitochondrial import in *Tim23*+/− mice is responsible for the phenotype described here, other components than Tim44p must be affected.

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