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Effects of overexpression of mitochondrial transcription factor A on lifespan and oxidative stress response in *Drosophila melanogaster*

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ABSTRACT

Mitochondrial transcription factor A (TFAM) plays a role in the maintenance of mitochondrial DNA (mtDNA) by packaging mtDNA, forming the mitochondrial nucleoid. There have been many reports about a function of TFAM at the cellular level, but only a few studies have been done in individual organisms. Here we examined the effects of TFAM on the *Drosophila* lifespan and oxidative stress response, by overexpressing TFAM using the GAL4/UAS system. Under standard conditions, the lifespan of TFAM-overexpressing flies was shorter than that of the control flies. However, the lifespan of TFAM-overexpressing flies was longer when they were treated with 1% H_2O_2. These results suggest that even though excess TFAM has a negative influence on lifespan, it has a defensive function under strong oxidative stress. In the TFAM-overexpressing flies, no significant changes in mtDNA copy number or mtDNA transcription were observed. However, the results of a total antioxidant activity assay suggest the possibility that TFAM is involved in the elimination of oxidative stress. The present results clearly show the effects of TFAM overexpression on the lifespan of *Drosophila* under both standard conditions and oxidative stress conditions, and our findings contribute to the understanding of the physiological mechanisms involving TFAM in mitochondria.

1. Introduction

Mitochondrial transcription factor A (TFAM) was firstly identified in human cells as a transcription factor for mitochondrial DNA (mtDNA) [1,2]. Recent studies suggested that TFAM is a core component of the mitochondrial transcription in mammals [3,4]. However, some studies indicated that TFAM might not be a core transcription factor, although TFAM stimulates transcription in mitochondria [5–7].

On the other hand, it has been known that TFAM binds to mtDNA in a nonspecific manner. It was demonstrated that TFAM exists in about 1000 molecules per one mtDNA molecule in human cells [8]. This amount is mostly enough to cover the entire region of mtDNA [9]. The knockdown of TFAM resulted in mtDNA depletion, and the reduction of mtDNA copy number caused the reduction of the TFAM level in mouse [10], *Drosophila* [5], chicken [11] and human [12]. These findings suggest that the levels of mtDNA and TFAM are interdependent, and that TFAM plays a critical role in the maintenance of mtDNA by packaging mtDNA, forming the mitochondrial nucleoid.

Mitochondria are the major source of reactive oxygen species (ROS) in cells. The accumulation of ROS induces oxidative damage to mtDNA, proteins and lipids, and it has been shown to contribute to the decline in physiological function of cells [13,14], a variety of diseases [15], and aging [16–19]. Some studies investigated the relationship between TFAM and oxidative stress [20–22] and showed that TFAM has some roles against oxidative stress; however, the relevance of these roles has not been established. Therefore, the clarification of the role of TFAM in a protective function against oxidative stress will contribute to our understanding of mitochondrial maintenance.

Many studies of TFAM function have been carried out using cultured cells, but only a few studies demonstrated the effects of TFAM when the TFAM expression level is changed in individual organisms. Here, we constructed transgenic flies overexpressing TFAM and examined the effects of its overexpression. *Drosophila* provides an attractive model for lifespan studies because of its short life cycle and the presence of orthologs of many human genes. *Drosophila* TFAM is 30 kDa, and it contains a mitochondrial-targeting signal, two high mobility group (HMG) motifs, a nu-

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clear-targeting signal and a C-terminal tail [23], which is similar to mammalian TFAM [2,24]. Through the HMG motifs, it is possible that *Drosophila* TFAM is able to package DNA, and thus to associate with the mtDNA–proteins complexes as shown in human TFAM [12,25].

In the present study, we demonstrated that excess TFAM has a negative effect on lifespan in *Drosophila*; however, it did not affect the mtDNA copy number or the transcriptional level of mtDNA. On the other hand, the lifespan of the TFAM-overexpressing flies was longer than that of the control flies under treatment with hydrogen peroxide (H_2O_2). These results suggest that TFAM plays a defensive role against oxidative stress in *Drosophila*.

2. Materials and methods

2.1. Fly strains

For overexpressing *Drosophila* TFAM, we used the GAL4/UAS system in *Drosophila melanogaster* [26]. To establish the transgenic UAS-*Tfam* line, we subcloned the coding region of the *Drosophila* TFAM into pUAST vector and introduced the construct into w^{1118} flies by the standard method. The detailed procedure will be described in a separate paper (Ibaragi et al., in preparation). By inverse PCR and sequencing, we confirmed that the *Tfam* transgene was inserted in the third chromosome (*w*;+/+;*UAS-Tfam-HA/TM3, Sb Ser*). We used the hs-N630 line as a GAL4 driver strain, and the F_1 heterozygotes (N630-GAL4/UAS-*Tfam*) were used in all experiments. For the control, we used the progeny from the cross between the w^{1118} flies without the *Tfam* transgene and the hs-N630 (N630/+). All flies were maintained at 25 °C in the standard *Drosophila* medium.

2.2. Quantitative RT-PCR

Total genomic DNA was extracted from 20 males by phenol–chloroform extraction. Total RNA was isolated from 10 females or 10 males using TRIzol® Reagent (Invitrogen), and after treatment with DNaseI, RNA was converted to cDNA using the SuperScript III First-Strand synthesis system (Invitrogen). Real-time quantitative reverse transcription-PCR (qRT-PCR) was carried out using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). The following primers were used for q-PCR: *Tfam*: 5′-CAGGGC GACAAGCAAACCTA-3′ and 5′-CGCGACTCCTGCATGTAGAC-3′, mtDNA (corresponding in position to 1445–1607 of mtDNA): 5′-CGCCTAAACTTCAGCCACTTAATC-3′ and 5′-AATGCTCCAGGATGTCC TAATTCA-3′, *COI*: 5′-TTTGACCCAGCGGGAGG-3′ and 5′-GTTTCC TTTTTTCCTGATTCTTGTCTA-3′, *ND1*: 5′-TAAAGCCAAACCCCCTCTT CTATA-3′ and 5′-CTGAAACTAATCGGAATCCTTTTGA-3′, *rp49*: 5′-GATGCCCAACATCGGTTACG-3′ and 5′-TTGTGCACCAGGAACTTCT TGA-3′. Thermocycling was conducted in the ABI 7300 Real-Time PCR System (Life Technologies Inc.) according to the manufacturer's protocol. The relative quantity of amplified DNA of each gene was calculated using the comparative Ct method and normalized to the expression of *rp49* in each sample.

2.3. Western blotting

Twenty males were homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma–Aldrich). Equivalent amounts of total proteins were separated by SDS–PAGE and then transferred onto nitrocellulose membranes. After blocking, the membranes were incubated with the primary antibody against *Drosophila* TFAM or β-tubulin (Developmental Studies Hybridoma Bank) for 1 h at room temperature. The production of antibody for the *Drosophila* TFAM will be described elsewhere (Ibaragi et al., in preparation). The membranes were then washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Signals were visualized by the ECL system (GE Healthcare).

2.4. Lifespan assay

Under the standard conditions, virgin females and males of the F_1 progeny from the crosses described in Section 2.1 were collected and maintained separately on the standard *Drosophila* medium at an initial number of 30 flies per vial at 25 °C. The flies were transferred to fresh vials every three days, and the numbers of dead flies were counted at the time of transfer until all flies were dead. Under oxidative stress conditions, 20 of the 3-day-old males were placed per vial and maintained on Kimwipe paper soaked with 5% sucrose containing 1% hydrogen peroxide (H_2O_2), or 5% sucrose without 1% H_2O_2 as a control at 25 °C. Before being placed in these vials, the flies were put into empty vials for starvation for 6 h. The flies were transferred to fresh vials every one or two days, and the numbers of dead flies were counted every day until all flies were dead. Survivorships were plotted, and the mean life spans were calculated as the age in days required to reach 50% survivorship.

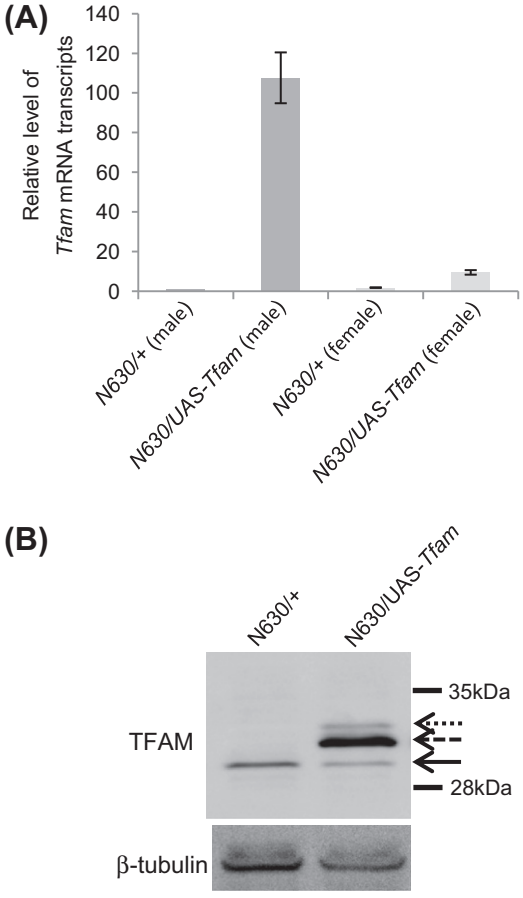

Fig. 1. Overexpression of *Tfam* transcript and TFAM protein in *Drosophila melanogaster*. (A) The transcription of *Tfam* in the TFAM-overexpressing flies (N630/UAS-*Tfam*) was significantly increased compared to that in the control flies (N630/+). The amount of *Tfam* transcript was normalized against the internal control, *rp49*, and the value obtained in the control males was assigned 1. Three samples were measured for each determination, and the mean ± S.E. is indicated. (B) TFAM protein was greatly increased in the TFAM-overexpressing males compared to that in the control males. β-tubulin was used as an internal control to show each equivalent amount of total proteins.

2.5. Total antioxidant assay

Total antioxidant activity was measured using the Total Antioxidant Power Kit (Oxford Biomedical Research). Twenty males were homogenized in phosphate-buffered saline (PBS), pH 7.0, and centrifuged at 3000*g* for 12 min at 4 °C, and the supernatants were then provided for the assay. The total antioxidant level is indicated as the capacity to convert Cu^{2+} to Cu^{+} by all of the antioxidants.

2.6. Statistical analyses

In the analyses of the transcriptional levels of *Tfam* and mtDNA, the mtDNA copy number, the lifespan assay and the total antioxidant assay, two-tailed Student's *t*-tests were performed.

3. Results and discussion

3.1. Overexpression of Drosophila TFAM

To investigate the function of TFAM within individual flies, we overexpressed TFAM using the GAL4/UAS system as described above, and the individuals' lifespan and oxidative stress responses were examined. The real-time qRT-PCR showed that the *Tfam* mRNA transcript level was significantly increased in the TFAM-overexpressing flies compared to the control flies: more than 100-fold in the males and about 5-fold in the females (Fig. 1A). It is not clear why the transcript levels were greatly different between the females and males. It was reported that GAL4-mediated expression was not detected in a female germ line [26]. This might be related to the present result, but further investigation is required before any hypothesis is made.

The Western blotting analysis using anti-*Drosophila* TFAM antibody confirmed the significant increase in the TFAM protein level in the TFAM-overexpressing flies (Fig. 1B). Three bands were observed in the TFAM-overexpressing flies. The band found in N630/+ and the corresponding band in N630/UAS-*Tfam* are from the endogenous TFAM. The other two bands in N630/UAS-*Tfam* are surmised to be derived from the UAS-*Tfam*; the upper band may be the immature TFAM with a mitochondrial-targeting signal, and the lower band may be the mature TFAM without it. The difference in molecular weight between the mature overexpressed TFAM and the endogenous TFAM is due to HA-tag.

3.2. The influence of TFAM overexpression on the lifespan

It was suggested that TFAM has a defensive function against stresses such as ROS by binding to mtDNA nonspecifically [27]. We therefore expected that the lifespan of the TFAM-overexpressing flies would be longer than that of the control flies. However, the mean lifespans of the TFAM-overexpressing females and males were 74.41 ± 1.10 days and 62.51 ± 0.47 days, respectively, whereas those of the control flies were 72.36 ± 1.25 days and 70.18 ± 1.04 days, respectively. The survivorship of the TFAM-overexpressing females was not different from the controls, whereas in the TFAM-overexpressing males, the survivorship was significantly shorter than that of the controls (Fig. 2A).

The results of the real-time qRT-PCR, which indicated that the increase in the transcript level of *Tfam* was significantly higher in the males than in the females (Fig. 1A), suggest that excess TFAM has a negative influence on lifespan in *Drosophila*. Although we used only one transgenic strain for overexpressing TFAM in the present study, it is not likely that the negative influence on lifespan

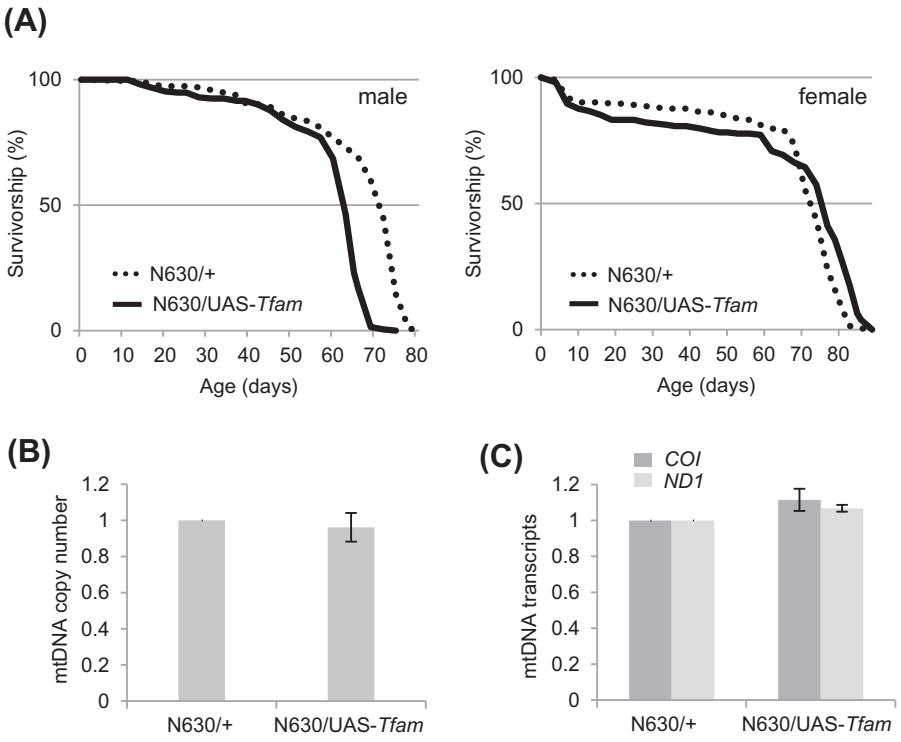

Fig. 2. Effects of TFAM overexpression on the lifespan, mtDNA copy number and mtDNA transcription under standard conditions. (A) The mean lifespan of the TFAM-overexpressing flies (N630/UAS-*Tfam*) was significantly shorter than that of the control flies (N630/+) in the males ($P < 0.05$). The total numbers of flies counted were 194 for N630/+ (male), 213 for N630/UAS-*Tfam* (male), 202 for N630/+ (female), and 202 for N630/UAS-*Tfam* (female). (B, C) For the mtDNA copy number (B) and the transcript levels of *COI* and *ND1* (C), no significant differences were detected between the TFAM-overexpressing flies and the control flies. Male flies at the age of 14 days were examined. Both the amount of mtDNA copy number and the transcript level were normalized against the internal control, *rp49*, and the value obtained in the control flies was assigned 1. Three samples were used for each determination, and the means ± S.E. are indicated.

in the males is due to the insertion of the transgene itself, since the lifespan of the females were not different from that of the controls.

To determine the factors affecting the changes in lifespan, we examined the mtDNA copy number and the mtDNA transcription level. The mtDNA copy number was estimated by real-time qRT-PCR, but no significant difference was detected between the TFAM-overexpressing flies and the control flies at the age of 14 days (Fig. 2B). As for the transcription, the transcript levels of both *COI* and *ND1*, encoded in different strands of *Drosophila* mtDNA, were not significantly different from the control flies in the 14-day-old TFAM-overexpressing flies (Fig. 2C).

Although some studies indicated that the overexpression of TFAM resulted in an increase of the mtDNA copy number in transgenic mice ubiquitously expressing human TFAM or in *Drosophila* cultured cells [28,29], another study demonstrated that the overexpression of TFAM caused a decrease in the mtDNA copy number in human cultured cells [30]. Regarding the mtDNA transcription, a previous report indicated that in human cultured cells it was increased when the TFAM level was low at the early stage of its overexpression, but it was decreased when the TFAM levels had doubled [31]. Similarly in *Drosophila*, the inhibition of mtDNA transcription by excess TFAM was also observed in cultured cells, and negative effects due to TFAM binding to the whole genome were pointed out [32]. Those authors also suggested that mitochondrial Lon protease regulates the amount of TFAM by a degradation of excess TFAM and contributes to the maintenance of the TFAM: mtDNA ratio for mtDNA biogenesis and homeostasis [32].

In the present results using *Drosophila* individuals, changes in the mtDNA copy number or transcription level were not detected when TFAM was overexpressed, although the lifespan of the TFAM-overexpressing flies was shorter than that of the control flies. Since the requirements for mitochondrial homeostasis might be different between cultured cells and whole bodies of *Drosophila*, the effects of excess TFAM might be different. The molecular mechanisms underlying the shortened lifespans of the individuals remain to be clarified.

3.3. The influence of TFAM overexpression on oxidative stress response

Since mitochondria are simultaneously the major source of both energy and ROS, it is possible that some proteins located in mitochondria are involved in a defensive function against ROS. Most TFAM proteins are located in mitochondria, and TFAM is a major component for forming the nucleoid structure [9]. It is thus thought that TFAM plays an important role in the responsive reaction against oxidative stress.

To investigate the effect of TFAM on the oxidative stress response in *Drosophila*, we used only male flies, since the transcript level of *Tfam* in TFAM-overexpressing flies was greatly increased in the males (Fig. 1A).

The lifespan assays were repeated twice. The mean lifespans of the TFAM-overexpressing males in Experiments 1 and 2 were 7.15 ± 0.23 days and 7.98 ± 0.42 days, respectively, whereas those of the control flies were 5.96 ± 0.13 days and 6.14 ± 0.19 days, respectively. Almost all of the flies died within about 10–12 days (Fig. 3A). These results demonstrated that the lifespan of the TFAM-overexpressing flies was significantly longer than the control flies under H_2O_2 treatment.

The number of mtDNA copy under H_2O_2 treatment was then estimated by real-time qRT-PCR. The results demonstrated that the changes in mtDNA copy number by treatment with H_2O_2 were not significantly different between the TFAM-overexpressing flies and the control flies (Fig. 3B), as was observed under the standard conditions (Fig. 2B). These findings imply that TFAM overexpression may mitigate H_2O_2-induced strong oxidative stress in *Drosophila* individuals.

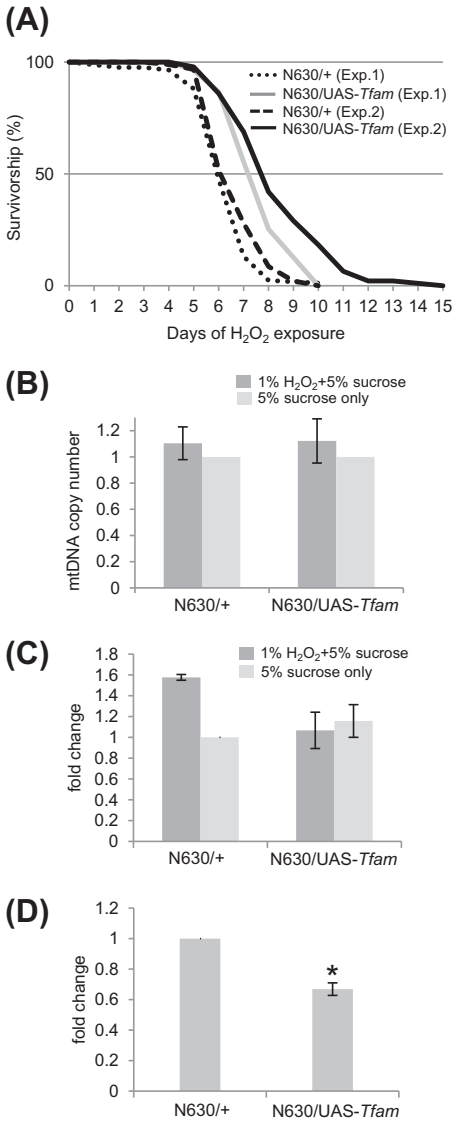

Fig. 3. The effects of TFAM overexpression on lifespan, mtDNA copy number and total antioxidant power under H_2O_2 treatment. (A) The mean lifespan of the TFAM-overexpressing males (N630/UAS-*Tfam*) was significantly longer than that of the control males (N630/+) ($P < 0.05$) in the repeated experiments. The total numbers of flies counted were 84 for N630/+ and 83 for N630/UAS-*Tfam* in Experiment 1, and 93 for N630/+ and 93 for N630/UAS-*Tfam* in Experiment 2. (B) No significant changes in the mtDNA copy number were detected between the TFAM-overexpressing flies and the control flies following treatment with H_2O_2. The amount of mtDNA copy number was normalized against the internal control, *rp49*, and the values obtained with 5% sucrose only were assigned 1 for the control flies and the TFAM-overexpressing flies. Three samples were used for each determination, and the means ± S.E. are indicated. (C) The total antioxidant levels, determined as the capacity to convert Cu^{2+} to Cu^{+}, was increased in the control flies, but that in the TFAM-overexpressing flies remained about the same under the H_2O_2 treatment. The value obtained in the control flies treated with 5% sucrose only was assigned 1. Two samples were used for each determination, and the means ± S.E. are indicated. (D) A significant difference in reduction activity was observed between the control flies and the TFAM-overexpressing flies under the H_2O_2 treatment ($P < 0.05$). The value obtained in the control flies was assigned 1. Four samples were used for each determination, and the means ± S.E. are indicated. In the experiments of (B), (C), and (D), male flies were treated with 5% sucrose containing 1% H_2O_2 or 5% sucrose only for three days, starting at the age of 3 days.

The above inference is consistent with previous studies that investigated the association between TFAM and oxidative stress in cultured cells. In SH-SY5Y cells, TFAM was reported to show a protective function against β-amyloid-induced oxidative damage [21]. Other studies showed that TFAM binds preferentially to

DNA containing 8-oxoguanine [33] and that TFAM modulates base excision repair against damaged mtDNA by its DNA binding activity and protein interactions [34].

Based on the present results, we hypothesized that TFAM participates in a degrading or eliminating mechanism for H_2O_2. Regarding the physiological responses to oxidative stress that is given from outside of the body, there are various reactions such as reduction and detoxification to reduce oxidative stress. To test whether TFAM overexpression was related to changes in the amounts of antioxidants, we performed a total antioxidant power assay. As shown in Fig. 3C, the control flies treated with H_2O_2 showed higher levels of reduction activity compared to the untreated flies. However, such an increase in reduction activity was not observed in the TFAM-overexpressing flies despite H_2O_2 treatment.

Additional measurements of the flies treated with H_2O_2 showed that the activity level of total antioxidant was significantly different between the control flies and the TFAM-overexpressing flies (Fig. 3D). This finding is quite interesting in that the reduction activity of TFAM-overexpressing flies was lower than that of the control flies despite the increase in their lifespan under H_2O_2 treatment. It may be that H_2O_2 is rapidly eliminated from the body in the TFAM-overexpressing flies compared to the control flies. We are currently investigating this possibility.

In conclusion, the present results clearly showed the effects of TFAM overexpression on the lifespan of *Drosophila* under both standard and oxidative stress conditions. Although excess TFAM was observed to have a negative effect on lifespan under the standard conditions, TFAM was suggested to function defensively against oxidative stress to increase the lifespan. Our results suggest a novel function of TFAM related to responsive reactions against strong oxidative stress.

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