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Short report

MICS-1 interacts with mitochondrial ATAD-3 and modulates lifespan in *C. elegans*

Michael Hoffmann [a,1], Sebastian Honnen [b,c,1], Ertan Mayatepek [a], Wim Wätjen [c], Werner J.H. Koopman [d], Olaf Bossinger [b], Felix Distelmaier [a,*]

[a] Department of General Pediatrics and Neonatology, University Children's Hospital , Heinrich-Heine-University, D-40225 Düsseldorf, Germany
[b] Institute of Molecular and Cellular Anatomy, RWTH Aachen University, D-52074 Aachen, Germany
[c] Department of Toxicology, Heinrich-Heine-University, D-40225 Düsseldorf, Germany
[d] Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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ABSTRACT

Caenorhabditis elegans open reading frame T21C9.1 encodes an uncharacterized protein, which is here named MICS-1 (mitochondrial scaffolding protein-1). It is predicted to be the homolog of human outer mitochondrial membrane protein 25 (OMP25 or synaptojanin-2-binding protein), which is a PDZ domain containing protein with a putative role in cellular stress response pathways. Here, we provide evidence that MICS-1 is an interacting partner of mitochondrial protein ATAD-3 (homologue of human ATAD3), which is essential for *C. elegans* development. We demonstrate that *mics-1(RNAi)* animals or *mics-1* mutants display enhanced longevity with an increased mean lifespan of up to 54% compared to control animals. Of note, also *atad-3(RNAi)* promoted longevity, although to a lesser extend (29% compared to controls). In addition, thermal stress of *mics-1* mutants induced low reactive oxygen species (ROS) production, whereas *atad-3(RNAi)* animals were highly sensitive to this assay, displaying drastically increased ROS levels. Further studies revealed that MICS-1 and ATAD-3 associated longevity was partially dependent on the presence of DAF-16. However, for both conditions, we also found a DAF-16 independent extension of lifespan. Finally, we observed an additional lifespan extension in *mics-1* mutants when subjected to *atad-3(RNAi)* whereas heat induced ROS production was even aggravated under this condition. This suggests (partially) independent effects of MICS-1 and ATAD-3 on lifespan and ROS production *in vivo*.

1. Introduction

Mammalian AAA domain-containing protein 3 (ATAD3) is a member of the AAA-ATPase family. ATAD3 is highly conserved among different species. It exists in *Caenorhabditis elegans* (*C. elegans*), *Arabidopsis thaliana* and *Drosophila melanogaster* as a single gene. In humans, three forms of ATAD3 have been identified and are documented in the NCBI database (http://www.ncbi.nlm.gov): a 66-kDa ATAD3A (BC033109; the ancestral form of the ATAD3 proteins), a 72.6-kDa ATAD3B (NM_031921) and a 46-kDa ATAD3C (NM_001039211) (Fang et al., 2010). Mitochondrial research identified ATAD3 in a proteomic analysis of mouse mitochondrial inner membrane proteins (e.g. TOB3; Da Cruz et al., 2003; Mootha et al., 2003). Later, an apparent role of ATAD3A in mitochondrial nucleoid organization was suggested and it was claimed to have the ability to bind mitochondrial DNA D-loop structures (He et al., 2007). However, follow-up studies demonstrated that a direct interaction of the protein with D-loop structures is unlikely (Bogenhagen et al., 2008). Recent studies on the topology of ATAD3A in mitochondrial membranes indicate that the N-terminal part of ATAD3A is outside the inner membrane and that the C-terminal part is inside the matrix. It was suggested that ATAD3A regulates dynamic interactions between the mitochondrial outer and inner membrane sensed by the cell fission machinery (Gilquin et al., 2010a). Moreover, an interaction between S100B, a calcium sensor protein, and ATAD3A was demonstrated, which is important for the cytoplasmic processing and subcellular localization of ATAD3A (Gilquin et al., 2010b).

Previously, we identified the *C. elegans* protein ATAD-3 as a homologue of mammalian ATAD3 (Hoffmann et al., 2009). We demonstrated that ATAD-3 is a mitochondrial protein, which is essential for *C. elegans* development. However, the mechanism behind these observations remained unresolved. Of note, ATAD-3 exhibits a class I PDZ binding motif at its C-terminus (−ETAV), which might be important for the interaction with other proteins.

Here, we provide evidence that ATAD-3 interacts with an uncharacterized PDZ domain containing protein, which is here named MICS-1 (mitochondrial scaffolding protein-1). We demonstrate that depletion of MICS-1 drastically extends lifespan and lowers thermal stress induced reactive oxygen species (ROS) production. Also ATAD-3

Abbreviations: MICS-1, mitochondrial scaffolding protein-1; OMP25, outer mitochondrial membrane protein 25; ROS, reactive oxygen species.

* Corresponding author. Department of General Pediatrics, Heinrich-Heine-University, Moorenstr. 5,D-40225 Düsseldorf, Germany. Tel.: +49 211 811 7640; fax: +49 211 811 8757.
E-mail address: felix.distelmaier@med.uni-duesseldorf.de (F. Distelmaier).

[1] These two authors contributed equally.

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depletion extended *C. elegans* lifespan, however heat stress induced ROS production was clearly increased in these animals. Further studies revealed that MICS-1 and ATAD-3 associated longevity was partially dependent on the presence of DAF-16. However, for both conditions, we also observed a DAF-16 independent extension of lifespan. Finally, *atad-3(RNAi)* in *mics-1* mutants additionally extended lifespan whereas stress induced ROS production was even aggravated in this condition.

2. Materials and methods

2.1. C. elegans strains and culture

Maintenance and handling of *C. elegans* were carried out as described previously (Brenner, 1974). Bristol N2 was used as the wild type strain. Single mutant strains were as follows: RB1335: *mics-1(ok1451)*, CB1370: *daf-2(e1370)*, CF1038: *daf-16(mu86)*. Transgenic strains were as follows: TJ356: integrated DAF-16::GFP roller strain (Henderson and Johnson, 2001).

2.2. Measurement of reactive oxygen species (ROS)

Measurements of heat stress induced ROS production was essentially performed as described by others (Kampkötter et al., 2007). In brief, 8–16 adult worms per trial were washed in TBST for 30 min and than individually transferred to the wells of a 384-well microtiter plate containing a final concentration of 12.5 μM 2,7-dichlorohydrofluorescein diacetate (H2DCF-DA; Molecular Probes Inc., Leiden, The Netherlands) in TBST. The measurement was performed in a Wallac 1420 Victor^2 multilabel counter (Perkin-Elmer, Monza, Italy) for 240 min at designated temperatures. Absolute fluorescence intensity values at 240 min were analyzed. In addition, data was validated by performing a linear fit for representative traces during a time span of linear increase of the fluorescence signal (according to Koopman et al., 2006). The slope of the fit was analyzed. Essentially, the same results were obtained as for the 240 min endpoint analysis.

2.3. RNA-mediated interference (RNAi)

RNAi by "feeding" was performed essentially as described by Kamath et al. (2001). The RNAi feeding clones for *atad-3* and *mics-1* were obtained from the Ahringer RNAi library (Geneservice Limited, UK). Clones were verified by sequencing. HT115(DE3) bacteria carrying the empty L4440 vector were used as controls.

2.4. Lifespan assay

Lifespan was determined at 25 °C according to a modified protocol of Wilson et al. (2006). Worms were transferred and grown on plates (three plates per trial) seeded with HT115(DE3) bacteria harboring the empty L4440 "feeding"-vector or L4440 with a fragment of the gene of interest (Brenner, 1974; Kamath et al., 2001; Timmons et al., 2001). Synchronized worms were transferred to fresh plates containing 25 μM FUDR (5-Fluoro-2′-deoxyuridine; Sigma–Aldrich) after reaching the young adult stage for 48 h. Subsequently, worms were transferred to new plates containing FUDR for additional 48 h (until the reproductive period was over). Next, worms were taken to plates without FUDR (Hosono, 1978; Tissenbaum and Guarente, 2001). Individuals were investigated at least every second day and considered as dead if no pharyngeal pumping was evident and they failed to respond to repeated gentle prodding. Individuals were counted and censored when dying upon "rupture" or "bag of worms" phenotypes or were untraceable. The resulting data sets of adult lifespan were analyzed using Kaplan–Meier survival test and weighted log-rank tests (Woolson and Clarke, 2002).

2.5. Protein–protein interactions

Co-Immunoprecipitation (Co-IP) and yeast 2-hybrid analysis were performed as described previously (Hoffmann et al., 2010). For Co-IP, 1 mg of protein lysate from either control or *atad-3(RNAi)* treated worms was preincubated with protein A agarose beads for 1 h at 4 °C on a rotating platform. Following centrifugation (12,000 rpm, 4 °C, 2 min), the supernatant was incubated with antibodies against ATAD-3 (rat, 1:1000; Hoffmann et al., 2009) for 2 h at 4 °C on a rotating platform. After addition and incubation with protein A agarose beads, beads were washed with TNT buffer three times. SDS–PAGE and western blot analyses were carried out as described by Hoffmann et al. (2009), using a cross reacting polyclonal antibody against human omp25 (rabbit, 1:1000; Nemoto and De Camilli, 1999) and anti-rabbit secondary antibody (HRP conjugated, 1:5000). For yeast 2-hybrid analysis, a 280 bp fragment encoding for the PDZ domain of MICS-1 was cloned into GAL4 DNA-binding domain vector pGBKT7 vector (Clontech), and a 650 bp c-terminal fragment of ATAD-3 was cloned into pACT2 vector (Clontech), either with or without the -ETAV motif. Empty pGBKT7 and pACT2 vectors were used as controls, respectively. As an additional negative control we used *Drosophila* Bazooka PDZ domain construct (Hoffmann et al., 2010).

2.6. DAF-16 translocation and dauer assays

For analysis of subcellular DAF-16 localization, synchronized L4 stage TJ356 were placed on HT115 or RNAi plates for 48 h. The strain TJ356 expresses a DAF-16::GFP fusion protein and can be used to monitor DAF-16 subcellular localization after heat stress (Henderson and Johnson, 2001; Kampkötter et al., 2008). After treatment at 37 °C for 30 min, worms were placed on microscope slides in 1 mM levamisole and capped with cover slips. DAF-16 subcellular distribution was analyzed on an Axiolab fluorescence microscope (Zeiss, Göttingen, Germany) at 100-fold magnification. Distribution of DAF-16::GFP was classified into two categories (cytosolic and translocated, i.e. nuclear/intermediate).

Dauer assay was performed as described elsewhere (Hu et al., 2006). In brief, gravid adults were put on individual 60 mm NGM-plates where we allowed egg-laying for 4–6 h at 20 °C before worms were removed again. Plates were shifted to the assay temperature of 27 °C. After 48–60 h the stages were scored for L1 diapause and dauers. Individuals larger than L2 larvae but not predauer/dauer stages were pooled as "other".

3. Results and discussion

Bioinformatic prediction (Chen et al., 2008) identified an uncharacterized protein, which is encoded by the predicted open reading frame T21C9.1, as a putative interaction partner of ATAD-3. The protein is homologous to the human outer mitochondrial membrane protein 25 (OMP25 or synaptojanin-2-binding protein), which is a PDZ domain containing protein with roles in cellular stress response pathways (Nemoto and De Camilli, 1999; Court et al., 2005).

To verify this prediction, we performed a yeast two-hybrid analysis, using the ATAD-3 C-terminal part, which confirmed the interaction of ATAD-3 with the above-mentioned protein (Fig. 1A; see also (Hoffmann et al., 2010)). Interaction was abolished if the PDZ domain-binding motif "-ETAV" in the ATAD-3 C-terminal part was depleted. We will further refer to this protein as MICS-1 (mitochondrial scaffolding protein-1), which was named according to general properties of PDZ domain proteins to promote clustering of target proteins at plasma membranes (Hughes and Fields, 1999).

Up to now, there exists very few information about MICS-1. Derry et al. (2007) included MICS-1 in a study about CEP-1 regulated genes in response to UV radiation. Severance et al. (2010) identified MICS-1 in a screen for genes essential for metazoan heme homeostasis.

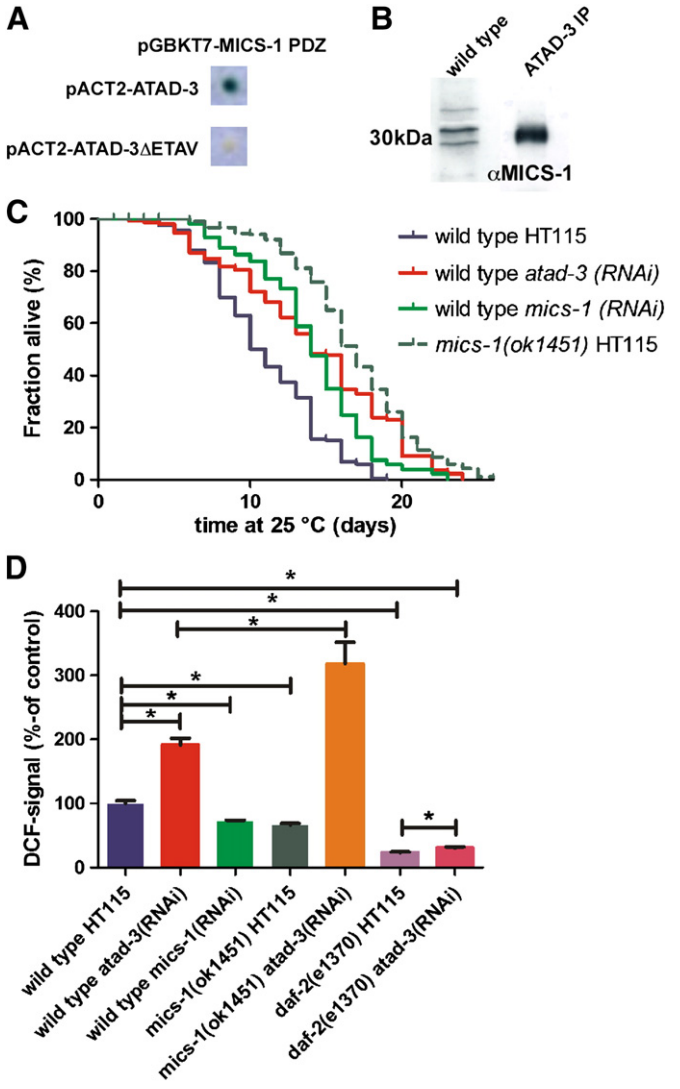

Fig.1. MICS-1 interacts with ATAD-3 and its depletion promotes longevity and lowers heat stress-induced ROS production in *C. elegans*. A) Yeast 2-hybrid analysis identifies an interaction of MICS-1 via the PDZ domain binding motif-ETAV in the C-terminal part of ATAD-3. B) Co-immunoprecipitations of ATAD-3 IPs, probed with MICS-1 antibodies, revealed a signal of the predicted size of MICS-1 protein at ~30 kDa in wild type lysate and ATAD-3 IPs. C) Survival plot of wild type HT115, *mics-1* HT115, wild type *mics-1(RNAi)* and wild type *atad-3(RNAi)*, respectively. Mean lifespan for *mics-1* HT115 was 16.9 +/− 0.2 days ($n = 270$) vs. 11 +/− 0.2 days ($n = 362$) for wild type animals ($p < 0.01$; Mantel–Cox log rank test). Mean lifespan for wild type *mics-1(RNAi)* was 14.1 +/− 0.3 days ($n = 233$; $p < 0.01$ compared to wild type). Mean lifespan for wild type *atad-3(RNAi)* was 14.2 +/− 0.4 days ($n = 274$; $p < 0.01$ compared to control). HT115 indicate the feeding RNAi *E. coli* strain carrying the empty feeding vector, used as control. D) Thermal induced reactive oxygen production (ROS) at 37 °C (expressed as percent of control). Asterisks indicate significant differences ($p < 0.05$) compared to control. In total, the following number of individual adult worms were analyzed in at least 2 independent trials: wild type HT115 ($n = 74$), wild type *atad-3(RNAi)* ($n = 50$), *mics-1* HT115 ($n = 35$), wild type *mics-1(RNAi)* ($n = 21$), *mics-1 atad-3(RNAi)* ($n = 16$), *daf-2(e1370)* HT115 ($n = 16$), *daf-2(e1370) atad-3(RNAi)* ($n = 19$). Statistical significance was calculated using unpaired two-tailed student's t-test.

However, no further details about the proteins function were provided. So far, to our knowledge, MICS-1 was not investigated in RNAi lifespan screens in *C. elegans*.

To further support our yeast two-hybrid analysis, we performed co-immunoprecipitations using ATAD-3 and MICS-1 antibodies (cross reacting antibody against OMP25; Nemoto and De Camilli, 1999). Incubation of protein lysates with ATAD-3 antibody and subsequent immunoblotting of the ATAD-3 bound proteins with MICS-1 antibody revealed a band at the predicted size of ~30 kDa (Fig. 1B). To further support the specificity of this result, we performed the experiment also in protein lysate of *atad-3(RNAi)* animals. Here, the 30 kDa band was absent (Suppl. Fig. 1).

To gain more information about the role of MICS-1 in *C. elegans* development, we performed *mics-1(RNAi)* by "feeding" (see also Hoffmann et al., 2009; Brenner, 1974). Moreover, we analysed *mics-1* mutant worms (strain RB1335 carrying the allele ok1451; provided by the Caenorhabditis Genetics Center).

In contrast to *atad-3(RNAi)* animals, *mics-1* mutants and RNAi animals developed normally and showed no growths or fertility defects. Moreover, these animals appeared healthy and active over a considerably longer time period than control worms. Accordingly, we performed a systematic lifespan analysis in *mics-1* mutants and RNAi animals. Assays were performed at 25 °C as described in the literature (modified according to Wilson et al. (2006)). As depicted in Fig. 1C, we observed an increase in lifespan when MICS-1 expression was reduced. In *mics-1* mutants, mean adult lifespan was increased by 54%, which is one of the most profound effects of a single gene on *C. elegans* longevity reported so far. Of note, our current systematic analysis also revealed increased longevity in *atad-3(RNAi)* worms, although to a lesser extend (29% compared to control; see also Table 1).

To further address the consequences of MICS-1 depletion *in vivo* and to test, whether the observed effects on lifespan were associated with an altered oxidative balance, we analysed reactive oxygen species (ROS) production under resting and heat stress conditions. Of note, increased ROS levels or oxidative stress are well known modulators of lifespan in *C. elegans* (Schulz et al., 2007). Assays were performed as described by others (Kampkötter et al., 2007; Koopman et al., 2006), using the fluorescent dye 2,7-dichlorohydrofluorescein diacetate (final concentration of 12,5 μM). The measurements were carried out for 240 min at 22 °C or 37 °C. At 22 °C, no enhanced ROS levels were measured (data not shown). However, under thermal stress at 37 °C, *mics-1* mutants and *mics-1(RNAi)* animals displayed considerably lower ROS levels compared to their control counterparts (Fig. 1D). In contrast, *atad-3(RNAi)* animals showed a drastic increase in heat induced ROS production, suggesting accelerated heat stress sensitivity.

Interestingly, in addition to this observation, we found that *atad-3(RNAi)* also increased ROS production under thermal stress conditions in *mics-1* mutants, suggesting that the ROS inducing effect of ATAD-3 depletion is independent of the presence of MICS-1 or even aggravated by MICS-1 depletion. Further evidence for the influence of ATAD-3 depletion on ROS production was delivered by experiments using *daf-2* mutant worms (Fig. 1D). DAF-2 is the insulin receptor homolog in *C. elegans*, which is capable to inactivate DAF-16 (Ogg et al., 1997). For *daf-2*

Table 1
Summary of lifespan data

Summary of life span (LS) measurements with standard error of the mean (SEM) for the different experimental conditions. HT115 indicate the feeding RNAi *E. coli* strain, carrying the empty feeding vector (Kamath et al., 2001; Timmons et al., 2001). Significant results are indicated by *($p < 0.01$; Mantel–Cox log rank test) with corresponding experiments in parenthesis.

	Background	Conditions	LS +/− SEM	n	Significance
1	Wild type	*HT115*	11 +/− 0.2	362	
2	Wild type	*atad-3(RNAi)*	14.2 +/− 0.4	274	*(1)
3	Wild type	*mics-1(RNAi)*	14.1 +/− 0.3	233	*(1)
4	Wild type	*daf-16(RNAi)*	8.9 +/− 0.2	142	*(1)
5	*mics-1(ok1451)*	*HT115*	16.9 +/− 0.2	270	*(1)
6	*mics-1(ok1451)*	*daf-16(RNAi)*	12.2 +/− 0.2	91	*(4)
7	*mics-1(ok1451)*	*atad-3(RNAi)*	20.2 +/− 0.2	130	*(5)
8	*daf-16(mu86)*	*HT115*	8.4 +/− 0.2	221	*(1)
9	*daf-16(mu86)*	*atad-3(RNAi)*	9.5 +/− 0.3	136	*(8)

mutants, in which DAF-16 displays higher activity, enhanced resistance to stresses has been documented (Honda et al., 2008). In our assay, ROS production under heat stress conditions was generally low in these animals but under *atad-3*(*RNAi*) conditions, ROS levels were slightly but also significantly increased (Fig. 1D). Taken together, results obtained in our heat stress assay indicated a general role of ATAD-3 in the regulation of heat stress induced ROS production.

Importantly, the transcription factor DAF-16 (homologue of Forkhead box O Transcription factors in mammals) is strongly involved in determining the rate of aging and average lifespan in *C. elegans*. DAF-16 selectively up-regulates genes that contribute to specific protective mechanisms, while simultaneously down-regulating potentially deleterious genes (Murphy, 2006). In view of the effects of MICS-1 deficiency on lifespan and ROS production, we tested in how far this effect was depended on the presence of DAF-16. We found that *daf-16(RNAi)* in *mics-1* mutants reduced lifespan extension from 54% down to 37% compared to *daf-16(RNAi)* animals (Fig. 2A, see also Table 1). This means, that the *mics-1* mutant longevity effect partially depends on the activity of DAF-16.

Comparably, lifespan modulating effects of *atad-3(RNAi)* were also partially dependent on DAF-16 (Fig. 2A). Nevertheless, in *daf-16(mu86)* submitted to *atad-3(RNAi)*, lifespan was still enhanced by about 13% if compared to *daf-16(mu86)* HT115 animals, suggesting also here a DAF-16 independent effect on *C. elegans* lifespan. At the current stage, it remains unclear, which additional transcription factors are involved in this phenomenon.

To complete our analysis of ATAD-3 and MICS-1 on aging, we performed *atad-3(RNAi)* in *mics-1* mutants. To our surprise, lifespan was enhanced to 83% if compared to wild type controls, and 19% if compared to the *mics-1* mutants (Fig. 2A, Table 1). This finding appeared unexpected in view of the increased heat stress-induced ROS production in this condition and suggests that the altered ROS levels might

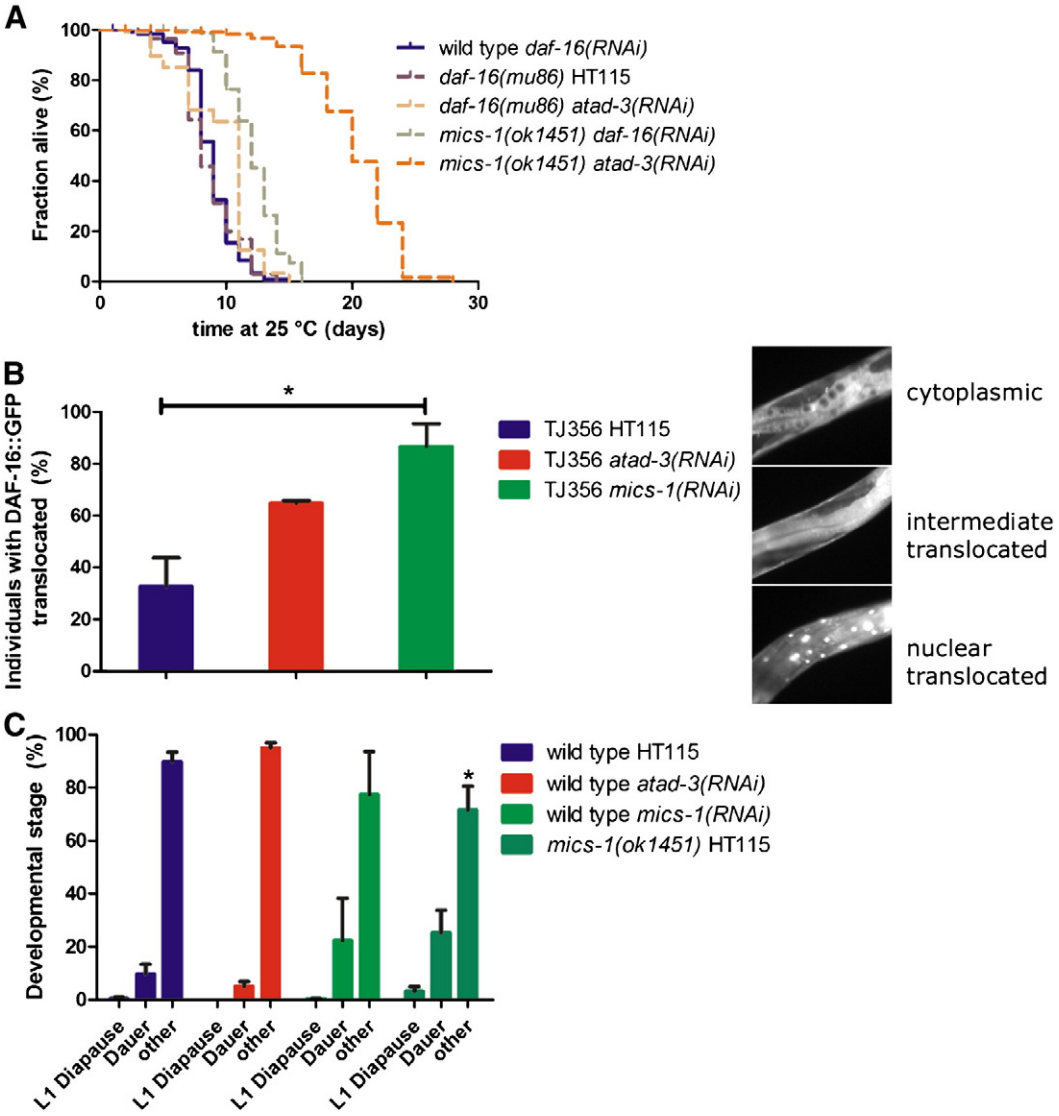

Fig.2. Lifespan extension in *mics-1* mutants is partially independent of the presence of DAF-16 and is additionally enhanced by *atad-3(RNAi)*. A) Survival plot of wild type *daf-16(RNAi)*, *daf-16(mu86)* HT115, *daf-16(mu86) atad-3(RNAi)*, *mics-1 daf-16(RNAi)* and *mics-1 atad-3*(RNAi). Mean lifespan for wild type *daf-16(RNAi)* was 8.9 +/− 0.2 days ($n = 142$), for *daf-16(mu86)* HT115 was 8.4 +/− 0.2 days ($n = 221$), for *daf-16(mu86) atad-3(RNAi)* was 9.5 +/− 0.3 days ($n = 136$), for *mics-1 daf-16(RNAi)* was 12.2 +/− 0.2 ($n = 91$) and for *mics-1 atad-3(RNAi)* was 20.2 +/−0.2 ($n = 130$). B) Strain TJ356 was used to demonstrate subcellular DAF-16 localization after heat stress in *atad-3(RNAi)* and *mics-1(RNAi)*. Typical images for cytoplasmic, intermediate and nuclear localization of DAF-16::GFP are depicted on the right. C) Dauer assays of wild type HT115, *mics-1* HT115, wild type *atad-3(RNAi)* and wild type *mics-1(RNAi)*. Asterisks indicate significant differences ($p < 0.05$) compared to control. Statistical significance was calculated using unpaired two-tailed student's t-test.

not be responsible for the modulation of lifespan, at least in MICS-1 depleted worms. This idea is further supported by the fact that ROS levels under resting conditions were not altered in *mics-1* mutants or *atad-3(RNAi)* animals.

Based on the observation that enhanced lifespan in MICS-1 depleted animals considerably depends on the presence of DAF-16, a higher DAF-16 activity might be suspected in these worms. To address this question, we analyzed the subcellular localization of DAF-16 by performing *mics-1(RNAi)* in TJ356 worms and subsequent microscopic analysis. This strain carries a transgene encoding a fusionprotein consisting of functional DAF-16 and the green fluorescent protein (GFP). It was widely used to study the localization of DAF-16 transcription factor under various conditions to give a hint about its activation status (Henderson and Johnson, 2001; Kampkötter et al., 2008). After subjecting MICS-1 depleted TJ356 worms to thermal stress, we found that the intermediate and nuclear translocation of DAF-16 was higher in *mics-1(RNAi)* than in control animals (Fig. 2B). In combination with our DCF-DA data, these results suggest a more efficient heat stress induced translocation of DAF-16 and consequent activation of ROS protective pathways in MICS-1 depleted animals.

To further support this finding, we performed dauer assays. DAF-16 activity is important for the efficient transition to dauer arrest at higher temperatures (Fielenbach and Antebi, 2008). In keeping with the above findings, our experiments revealed that a significantly lower amount of *mics-1* mutant animals did not arrest as L1 diapause or in dauer stage under heat stress conditions, further suggesting a higher activity of DAF-16 in MICS-1 depleted worms.

In parallel to the above experiments, we also tested DAF-16 activity in *atad-3(RNAi)* treated animals. We found that under similar conditions, nuclear localization of DAF-16 was not significantly altered in these worms (Fig. 2B), and also the rate of L1 diapause/dauer formation was comparable to controls (Fig. 2C). These results suggest that *atad-3(RNAi)* effects are not directly modulated by DAF-16.

In conclusion, our *in vitro* data suggest that MICS-1 is a novel interacting partner of mitochondrial ATAD-3. Depletion of MICS-1 clearly promotes *C. elegans* longevity and lowers heat stress induced ROS production. Lifespan prolonging effects of MICS-1 were partially dependent on DAF-16. In line with this observation, DAF-16 displayed a higher degree of nuclear translocation in *mics-1(RNAi)* worms or *mics-1* mutants and animals showed an increased capability of entering the L1 diapause/dauer stage. On the other hand, ATAD-3 depletion also extended *C. elegans* lifespan and clearly increased heat induced ROS production. Also here, lifespan prolonging effects were partially dependent on DAF-16. Nevertheless, for MICS-1 and ATAD-3 deficiency, a considerable DAF-16-independent effect on lifespan was observed. This is certainly interesting and it will be an important next step to further investigate, which transcription factors and signalling pathways are responsible for this phenomenon. Surprisingly, we observed an additional lifespan extension in *mics-1* mutants when subjected to *atad-3(RNAi)* whereas heat induced ROS production was even aggravated under this condition. This suggests at least partially independent effects of MICS-1 and ATAD-3 on lifespan and ROS production.

Supplementary materials related to this article can be found online at doi:10.1016/j.exger.2011.12.011.

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