Original Article

ALKB-8, a 2-Oxoglutarate-Dependent Dioxygenase and S-Adenosine Methionine-Dependent Methyltransferase Modulates Metabolic Events Linked to Lysosome-Related Organelles and Aging in *C. elegans*

(lifespan / lysosome-related organelle / methyltransferase / multifunctional enzyme / Nile red / oxidoreductase, RNA-binding / transferase)

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Abstract: ALKB-8 is a 2-oxoglutarate-dependent dioxygenase homologous to bacterial AlkB, which oxidatively demethylates DNA substrates. The mammalian AlkB family contains AlkB homologues denominated ALKBH1 to 8 and FTO. The *C. elegans* genome includes five AlkB-related genes, homologues of ALKBH1, 4, 6, 7, and 8, but lacks homologues of ALKBH2, 3, and 5 and FTO. ALKBH8 orthologues differ from other AlkB family members by possessing an additional methyltransferase module and an RNA binding N-terminal module. The ALKBH8 methyltransferase domain generates the wobble nucleoside 5-methoxycarbonylmethyluridine from its precursor 5-carboxymethyluridine and its (R)- and (S)-5-methoxycarbonylhydroxymethyluridine hydroxylated forms in $tRNA^{Arg}_{UCG}$ and $tRNA^{Gly}_{UCC}$. The ALKBH8/ALKB-8 methyltransferase domain is highly similar to yeast TRM9, which selectively modulates translation of mRNAs enriched with AGA and GAA codons under both normal and stress conditions. In this report, we studied the role of *alkb-8* in *C. elegans*. We show that downregulation of *alkb-8* increases detection of lysosome-related organelles visualized by Nile red *in vivo*. Reversely, forced expression of *alkb-8* strongly decreases the detection of this compartment. In addition, overexpression of *alkb-8* applied in a pulse during the L1 larval stage increases the *C. elegans* lifespan.

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Abbreviations: AlkB – *Escherichia coli* α-ketoglutarate-dependent dioxygenase, ALKBH1 – alkylated DNA repair protein AlkB homologue 1, ALKBH8 – alkylated DNA repair protein AlkB homologue 8 (a.k.a. ABH8), *alkb-8* – *C. elegans* gene coding for the orthologue of ALKBH8, ALKB-8 – *C. elegans* orthologue of ALKBH8, CAN – canal-associated neuron, FTO – fat mass and obesity associated protein, GFP – green fluorescent protein, IPTG – isopropyl β-D-1-thiogalactopyranoside, LRO – lysosome-related organelles, qPCR – quantitative PCR, RNAi – RNA interference, RRM – RNA recognition motif, SAM – S-adenosyl methionine, TRM9 – yeast TRna methyltransferase 9, a SAM-dependent methyltransferase, TRX1 – thioredoxin.

Introduction

The 2OG/Fe(II) (2-oxoglutarate- and Fe^{2+}-dependent) oxygenase superfamily possesses an important position among oxygenases. The haem group is substituted in these enzymes by a protein module that coordinates Fe^{2+} and whose enzymatic activity is dependent on 2-oxoglutarate, which serves as an electron donor and is consumed during the enzymatic reaction while converted to succinate and carbon dioxide. Unlike monooxygenases, which are dependent on haem and which transfer one oxygen atom to the substrate and reduce the other oxy-

gen atom to water, 2OG/Fe(II) oxygenases incorporate both atoms of molecular oxygen (O_2) into the product(s) of the reaction and are classified as dioxygenases. 2-Oxoglutarate is a rate-limiting factor for the enzyme catalytic activity by its critical intracellular concentration level. Enzymes of this category function in a wide spectrum of metabolic processes including posttranslational modification of proteins, DNA repair, epigenetic modification of DNA, and regulation of hypoxia responsive genes (Aravind and Koonin, 2001; van den Born et al., 2011; Fedeles et al., 2015).

The AlkB family of dioxygenases encompasses homologues of AlkB from *Escherichia coli*, which is a DNA repair enzyme demethylating methylated DNA and RNA bases (e.g., 1-methyladenine and 3-methylcytosine). Mammalian AlkB homologues include nine genes, named *ALKBH1* to *8*, and a gene encoding fat mass and obesity associated protein (FTO) originally identified as a gene localized at a chromosomal locus associated with the rat fused-toes phenotype (Peters et al., 1999; Gerken et al., 2007; Fedeles et al., 2015). The *FTO* gene received attention for its association with human obesity (Frayling et al., 2007; Yajnik et al., 2009), later in part shown to be associated with homeobox gene *IRX3*, which is regulated by noncoding sequences within the *FTO* gene (Smemo et al., 2014). This connection is conserved between fish and mammals. Besides that, FTO has its own role in obesity as its global overexpression leads to hyperfagia and obesity (Church et al., 2010).

ALKBH8 homologues have a special position among all AlkB proteins for possessing two extra domains in addition to the dioxygenase domain, a methyltransferase domain and an N-terminal RNA recognition motif, which likely helps the AlkB domain in search for specifically modified tRNAs (Songe-Moller et al., 2010; Pastore et al., 2012). ALKBH8 has been shown to regulate the rate of protein synthesis from mRNAs that are coded by codons for which there is a limited amount of tRNA through modification of bases in the anti-codon region of tRNA, especially the wobble base, the first base in the anti-codon place of tRNAs, which following this modification can recognize additional codons (Songe-Moller et al., 2010; van den Born et al., 2011). ALKBH8 was shown to have a role in urothelial carcinoma cell survival mediated by NOX-1-dependent ROS signals. Silencing of ALKBH8 induced JNK/p38/γH2AX-mediated cell death (Shimada et al., 2009). The role of human ALKBH8 as a tRNA methyltransferase required for wobble uridine modification and DNA damage survival is well documented. Fu at al. (2010a,b) showed that the AlkB domain of mammalian ALKBH8 catalyses hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA. The AlkB domain of ALKBH8 specifically hydroxylates mcm(5)U into (S)-mchm(5)U diastereomer in tRNA-Gly(UCC) (van den Born et al., 2011).

The ALKBH8 methyltransferase domain shows close relationship to yeast methyltransferase TRM9. The function of the yeast TRM9 has been investigated (Kalhor and Clarke, 2003; Deng et al., 2015). The enzyme catalyses methylation of the wobble bases at position 34 in tRNA. U at this position can recognize all four bases, while the modified uridine residues are more restrictive and limit the recognition to only A and G, or to only one of these residues. Codon-biased translation can be regulated by wobble base tRNA modification systems during cellular stress responses (Chan et al., 2010, 2012; Gu et al., 2014). This mechanism is conserved in plants. In *Arabidopsis thaliana*, the Trm9 orthologue (AtTRM9, AT1G31600) and two other ALKBH8-like proteins AtTRM112a and AtTRM112b function in the formation of modified wobble uridines. AtTRM9 is responsible for the final step in mcm(5)U formation. The enzymatic activity of AtTRM9 depends on either AtTRM112a or AtTRM112b. *A. thaliana* ALKBH8 orthologue AtALKBH8 is required for hydroxylation of mcm(5)U to (S)-mchm(5)U in tRNA(Gly)(UCC). Plants with mutant *atalkbh8* have increased levels of mcm(5)U and of mcm(5)Um, its 2'-O-ribose methylated derivative, suggesting that accumulated mcm(5)U is prone to further ribose methylation by another mechanism (Leihne et al., 2011). Protozoan ALKBH8 oxygenases display both DNA repair and tRNA modification activities (Zdzalik et al., 2014).

ALKBH8 was shown to regulate selenocysteine-protein expression as a protective mechanism against damage by reactive oxygen species (Endres et al., 2015). *C. elegans* has two thioredoxin reductases, TRXR-1 and TRXR-2 (Buettner et al., 1999), but only one of them, TRXR-1, is a selenoprotein. Thioredoxin (TRX-1) is related to lifespan regulation and oxidative stress response in *C. elegans* (Jee et al., 2005; Miranda-Vizuete et al., 2006). TRXR-1 and TRXR-2 have differential physiological roles and localizations in *C. elegans*. TRXR-1 is a cytosolic protein. TRXR-2 is located in mitochondria, where reactive oxidative species are mainly generated, and protects mitochondria from oxidative stress, while cytosolic TRXR acts to maintain an optimal oxido-reductive status in the cytosol. The cytosolic *trxr-1* is highly expressed in the pharynx, vulva, and intestine. *trxr-2* is mainly expressed in pharyngeal and body wall muscles and its defects cause a shortened lifespan and a delay in development under stress conditions. Deletion mutation of selenoprotein *trxr-1* results in decreased acidification of the lysosomal compartment in the intestine. Interestingly, the acidification defect of *trxr-1* (jh143) deletion mutant was rescued, not only by selenocysteine-containing wild-type TRXR-1, but also by a cysteine-substituted mutant TRXR-1. Both *trxr-1* and *trxr-2* were upregulated when worms were challenged by environmental stress such as heat shock (Li et al., 2012).

A prominent feature of *C. elegans* enterocytes are lysosome-related organelles (LRO) called gut granules. Similarly as mature lysosomes, gut granules have internal acidic pH, contain hydrolytic enzymes and lack mannose-6-phosphate receptors. Gut granules are highly heterogeneous when analysed by electron microsco-

py, display various levels of birefringence in light microscopy and autofluorescence, which increases with animal age. In *C. elegans*, staining by Nile red applied on animals *in vivo* together with bacterial food allows highly reproducible functional determination of a specific subpopulation of lysosome-related organelles (Soukas et al., 2013). *In vivo* Nile red uptake may be used as an effective tool for identification of proteins that function at the level of specific LRO (Soukas et al., 2013).

In this report, we attempted to functionally characterize ALKB-8 in *C. elegans*. We show that *alkb-8* downregulation by RNAi leads to slightly accelerated larval development and elevated values of *in vivo* Nile red compartment staining. The forced expression of *alkb-8* downregulates this subcellular compartment. While downregulation of *alkb-8* does not affect *C. elegans* longevity, forced expression of *aklb-8* increases the *C. elegans* lifespan by approximately 30 %.

Material and Methods

Maintenance of C. elegans strains and transgenic lines

All *C. elegans* strains were maintained as described (Brenner, 1974). The-wild type strain N2 (var. Bristol) was obtained from the *C. elegans* Stock Center (https://cgc.umn.edu/).

Transgenic lines were prepared by microinjections of plasmid DNA into gonads of young adult N2 hermaphrodites using an Olympus IX70 microscope equipped with the Narishige microinjection system (Olympus, Tokyo, Japan). Injections were done as described (Fire et al., 1998; Tabara et al., 1999).

Synchronized populations of L1 larvae were prepared by the "Bleaching" technique, in which the cultured nematodes are treated with alkaline hypochlorite solution destroying all larval stages except the embryos that are protected by egg shells. Embryos hatch in liquid solution without access to food, which prevents further development. The protocol is described in Porta-de-la-Riva et al. (2012).

Isolation of genomic DNA

The genomic DNA used as a template for PCR reactions was isolated using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). For the isolation, we used about 50 mg of washed wild-type animals of mixed developmental stages and we followed the manufacturer's protocol "Isolation of Nucleic Acids from Mammalian Tissue."

Total RNA isolation and cDNA synthesis

Total RNA was isolated from N2 animals kept on 2% agarose plates. Animals of the required developmental stage and feeding status were washed with water, pelleted by centrifugation for 5 min at 200 x *g* and frozen at –80 °C. The frozen pellet was quickly melted and resuspended in 0.5 ml of resuspension buffer (0.5% SDS; 5% 2-mercaptoethanol; 10 mM EDTA; 10 mM Tris/HCl (pH 7.5) with 12.5 µl of proteinase K (20 mg/ml)), vortexed for 1 min and incubated for 60 min at 55 °C. RNA was isolated by phenol-chloroform extraction and ethanol precipitation and the pellet was dissolved in water. The sample was then treated with 1 unit of DNase I (New England Biolabs, Ipswich, MA) per 1 µg of total RNA for 30 min at 37 °C and purified by phenol-chloroform extraction and ethanol precipitation followed by RNA resuspension in DEPC water.

Complementary DNA (cDNA) was prepared with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) with random hexamers, according to the manufacturer's protocol.

Quantitative PCR

Total RNA from individual developmental stages (embryos, L1, L2, L3, L4, and young adult animals) and from fasted and fed animals was isolated and used for cDNA synthesis as described above. Quantitative PCR (qPCR) was performed using the Universal Probe Library technique (Roche Molecular Systems, Inc., Pleasanton, CA). Primers and probes were designed with the Universal Probe Library System Assay Design Software. Reactions were run in LightCycler 2.0 with software LightCycler 4.1 (Roche Molecular Systems), and the protocol described earlier (Vohanka et al., 2010) was used. The expression was normalized against *ama-1*. All samples were run in triplicates. The expression ratio (ΔΔCt) was calculated using the efficiency corrected model. In different developmental stages, the resulting values mean the fold change of expression compared to the expression in embryos. In fasting experiments, the result represents the fold change of expression compared to the fed control animals.

RNA interference

To downregulate the *alkb-8* expression, we used the RNAi feeding method, in which animals are fed on bacteria producing dsRNA, as previously described (Timmons et al., 2001).

To prepare the feeding vector, we first cloned the whole cDNA sequence of *alkb-8* into pCR®II vector using TA Cloning® Kit Dual Promoter (pCR®II) (Invitrogen). Primers used for the PCR reaction were 11/08 (5′ ATGTATTTCAATGAAGAAAAAGCGA 3′) and 10/08 (5′ TCAAATTTTCTTCGCAATAATAATA-TAA 3′). Then the *alkb-8* sequence was re-cloned into the L4440 vector using enzymes *Hind*III and *Xba*I. The *E. coli* strain HT115 was transformed with *alkb-8*::L4440 and empty L4440 control vector, and one colony of each was inoculated to LB medium with ampicillin (100 µg/ml final concentration) and let grow to $OD_{600} \approx 0.4$. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.4 mM to induce dsRNA production. The culture was grown for 4 h at 37 °C, and then 300 µl was plated onto NGM plates supplemented with ampicillin (100 µg/ml

final concentration) and IPTG (0.4 mM final concentration). The plates were kept at room temperature overnight, and the next day synchronized L1 larvae were placed on these plates.

Nile red staining

To estimate the LRO compartment visualization by *in vivo* Nile red uptake and resulting fluorescence, the synchronized L1 larvae (control larvae, larvae inhibited for *alkb-8* by RNAi or larvae with forced overexpression of *alkb-8*) were transferred on feeding culture + 50 ng Nile red/ml of culture. Three hundred µl of OP50 culture with Nile red was used per plate. Nematodes were kept at 22 °C for 48 h and then fluorescent pictures of young adults were acquired using identical settings and exposure times (magnification with 20× objective, exposure time 10 ms in RNAi experiments and 50 ms in overexpression experiments). Resulting images were analysed using the ImageJ program (https://imagej.net/). The total pixel intensity of the cytoplasmic area of the first two intestinal cells in images yielding highest fluorescence was determined and used for comparison.

Overexpression of alkb-8

The entire cDNA sequence of *alkb-8* was re-cloned from the pCRII vector into the expression vectors that contain heat-shock inducible promoters pPD49.78 and pPD49.83 using restriction enzymes *Eco*RV and *Kpn*I. Constructs were injected into N2 hermaphrodites (at a concentration of 50 ng/µl) along with a positive selection marker, pRF4 plasmid (50 ng/µl), which encodes a mutant collagen (*rol-6*(su1006)) that induces a dominant "roller" phenotype. As a control, we used animals injected only with the pRF4 plasmid.

Forced expression was induced in a synchronized population of L1 animals. Larvae were placed on plates seeded with OP50 bacterial culture and left for 2 h at RT for recovery, and then subjected to 30 min heat shock at 34 °C, after which the animals were kept at 22 °C and the lifespan was determined. In the Nile red staining experiment, the bacterial culture was supplemented with 15 ng of Nile red per plate. Pictures were taken after 50 h using a constant setting.

Preparation of the alkb-8::gfp transgene regulated by CEOP3136 promoter and endogenous 3' UTR

According to WormBase (release WS263), *alkb-8* is organized in a hybrid operon, CEOP3136. This operon includes four genes, *wdr-5*, *dph-1*, *alkb-8,* and *nrde-1*. Since the expression from a transgene regulated by the internal *alkb-8* promoter is already known, we constructed an expression vector to prepare a transgene expressing ALKB-8 tagged with green fluorescent protein (GFP) under the regulation of operon promoter and *alkb-8* endogenous 3' UTR. To achieve this, four amplified DNA fragments containing the operon promoter, *alkb-8* genomic sequence, gene coding for GFP and the 3'UTR of *alkb-8* were amplified (primer sequences are listed in Table 1) and assembled using GENEART® Seamless Cloning and Assembly Kit (Invitrogen) according to the manufacturer's protocol. The resulting product was verified by sequencing and used for preparation of transgenic lines (injected in a concentration of 50 ng/µl without pRF4 vector). The scheme of the construct is shown in Fig. 1**.**

Developmental assay

To estimate the timing of larval development of control *C. elegans* and animals with *alkb-8* downregulated by RNAi, a synchronized population of L1 larvae was prepared, and equal volumes of liquid larval culture were transferred to control plates containing HT115 bacteria with empty L4440 plasmid and plates containing bacteria transfected with the same plasmid but containing the cloned insert of *alkb-8*. Both bacterial cultures were induced by IPTG. The experiment was done in quadruplicate, from which one representative set was selected for more specific analysis. Equal surface of plates with experimental (photographed first) and control animals was photographed at the time when control animals started to first lay eggs (after 78 h at 16 °C), and the number of animals and laid eggs was determined in the photographs. The pictures were taken in an Olympus SD30 microscope (Olympus, Tokyo, Japan) with a Panasonic DMC-TZ3 camera (Panasonic, Kadoma, Japan).

Table 1. Primers used for seamless cloning and assembly of the $P_{CEOP3136}$::alkb-8::gfp::alkb-$8_{3'UTR}$ construct

Primer	sequence 5′→ 3′		Note
11/80	AATTCGAGCTCGGTACGGATAAGGAAGATCATCAATGTTT	S	CEOP3136 promoter
11/81	TCACACATATCTGAAATCACAGCAAAAATCAA	AS	CEOP3136 promoter
11/82	TTTCAGATATGTGTGAGTTCATTTTTCAACCC	S	*alkb-8* gDNA
11/83	GGGTCCTCAATTTTCTTCGCAATAATAATATA	AS	*alkb-8* gDNA
11/84	AGAAAATTGAGGACCCTTGGAGGGTACCGGTA	S	*gfp*
11/85	TAAAAAAACTATTTGTATAGTTCATCCATGCC	AS	*gfp*
11/86	ACAAATAGTTTTTTTAAAGTTTTTTCTATTGG	S	*alkb-8* 3'UTR
11/87	GCCAAGCTTGCATGCCTTTAGCGCAGTTTGAGAATCTGAA	AS	*alkb-8* 3'UTR

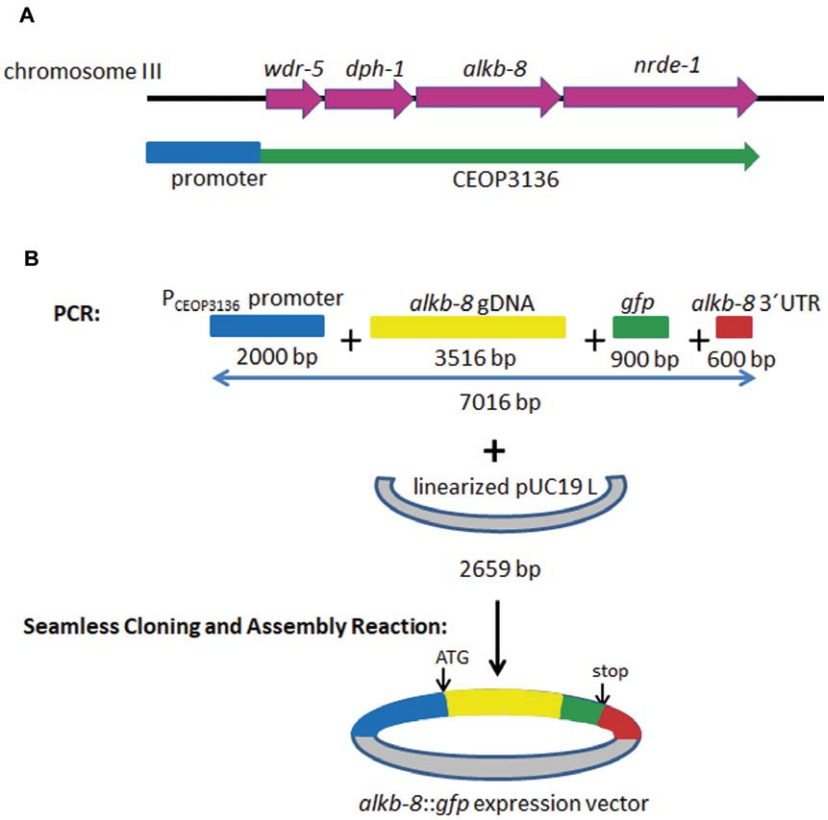

Fig. 1. Preparation of the transgenic line expressing *alkb-8::gfp* under the regulation of promoter of CEOP3136 and endogenous 3' UTR

A. Organization of *alkb-8* on chromosome III. *alkb-8* is the third gene in operon CEOP3136 and has its own internal promoter.

B. Strategy for preparation of transgene expressing ALKB-8 tagged by GFP at its 3' end. Corresponding fragments of the CEOP3136 promoter, *alkb-8* genomic sequence, gene coding for GFP based on pPD95.75 and *alkb-8* 3'UTR were amplified by PCR and assembled by the Seamless Cloning Assembly Reaction.

Lifespan determination

To determine the lifespan, a large scale of synchronized N2 L1 larvae was prepared and divided to control cultures and cultures subjected to *alkb-8* dsRNA produced by bacteria that were fed to experimental animals and synchronized populations of transgenes containing the *rol-6* gene as a control and experimental animals carrying extrachromosomal arrays containing *rol-6* and *alkb-8* cloned in heat-shock vectors pPD49.83 and pPD49.78. For each experimental condition, 100 L1 larvae were selected and followed on a daily basis throughout their complete lifespan in the overexpression experiment. In RNAi experiments, 60 animals were followed in each group.

Microscopy

Nomarski optics microscopy and fluorescence microscopy pictures were taken with an Olympus BX60 microscope equipped with a DP30BW CD camera (Olympus). Confocal microscopy was done using an inverted Leica SP8 TCS SMD FLIM system equipped with a 63 × 1.2 NA water immersion objective, a pulsed white light laser (470-670 nm), AOBS, and two internal hybrid single photon counting detectors and operated by the Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany).

Results

The expression of alkb-8 continues from embryonic stages through larval development to adults

We analysed the gross expression of *alkb-8* during developmental stages using reverse transcription-quantitative PCR. The results were normalized to the expression of polymerase II subunit *ama-1* and related to the expression observed in mixed stage embryos. The relatively high expression of *alkb-8* in the embryos decreased in synchronized L1 larvae and steadily increased from the L2 stage to young adults (Fig. 2A). We analysed the effect of 6 h fasting and feeding in synchronized L1 larvae at the level of expression of *alkb-8* (and *fat-7* and *asc-2*, for which the response to starvation is known). Starvation had no effect on *alkb-8* expression,

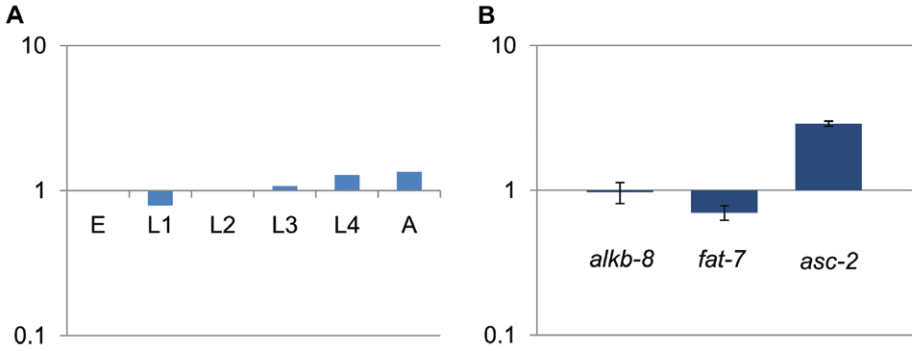

Fig. 2. The expression profile of the *alkb-8* gene analysed by RT-qPCR
A. The expression of *alkb-8* during development. Results are shown in logarithmic scale and the values represent the fold change of expression compared to the expression in embryos. The expression drops in the L1 stage and gradually increases during development.
B. The relative expression of *alkb-8* after six hours of fasting. The values represent the fold change of expression compared to the fed control animals. The expression of *alkb-8* is not affected by the feeding status. Genes previously reported to be affected by fasting (Van Gilst et al., 2005), *fat-7* (decreased expression after fasting) and *asc-2* (increased expression), were used for control.

while *fat-7* and *asc-2* expression responded to fasting as expected.

Tissue- and cell-specific expression of alkb-8 from the operon promoter

According to WormBase (release WS263), *alkb-8* is organized as a third gene in hybrid operon CEOP3136, indicating that its expression depends partially on the operon promoter and partially on its own promoter. The expression of *alkb-8* dependent on the internal promoter was described by Pastore et al. (2012) and revealed *alkb-8* expression decreasing during later larval stages, and the expression pattern was restricted to a small number of cells, especially several neurons. To visualize the *alkb-8* expression dependent on the operon promoter, we prepared lines carrying extrachromosomal arrays containing the transgene consisting of the CEOP3136 promoter, *alkb-8* genomic sequence fused to *gfp* and followed by the endogenous *alkb-8* 3'-UTR. The transgene is expressed ubiquitously in embryos from approximately the 40-cell stage throughout the embryonic development. The expression continued in L1 larvae, although it was necessary to use longer exposure time for its visualization in accordance with the decreased expression observed in L1 larvae in the RT-qPCR experiment. The cytoplasmic expression of the transgene was strong in neurons, pharyngeal and body wall muscles, and other tissues such as somatic gonad and the egg-laying apparatus (Figs. 3 and 4). We also observed diffuse expression in intestinal cells (Fig. 3).

The effects of alkb-8 downregulation and forced overexpression on C. elegans development

Downregulation of *alkb-8* by RNAi using the protocol with bacteria producing dsRNA did not reveal any directly observable phenotype. In contrast, the larvae with downregulated *alkb-8* seemed to be in a very good feeding status and possibly slightly bigger than the controls fed with bacteria containing empty vector expressing short non-specific dsRNA. Since the observed difference did not cause delays in complete larval stages, we analysed the onset of egg laying in control and RNAi-treated cultures. This strategy revealed a clearly observable difference in time given by the onset of egg laying by control larvae at which the larvae with downregulated *alkb-8* had already laid approximately 50 times more embryos (Fig. 5). No specific developmental defects were observed after forced overexpression of *alkb-8*.

The effect of alkb-8 downregulation and forced overexpression on the visualization of the Nile red-positive compartment

To assess the possible involvement of ALKB-8 in the function of lysosome-related organelles, we assayed the uptake of Nile red delivered to nematode synchronized cultures together with bacterial food. Animals with inhibited *alkb-8* showed markedly higher Nile red-dependent fluorescence in enterocytes. In both experimental and control animals, the Nile red fluorescence was higher in proximal enterocytes compared to enterocytes of the middle part of the gut. We therefore analysed the fluorescent signal in the first two proximal enterocytes. Densitometric analysis of Nile red-dependent fluorescence confirmed an approximately 30% increase of the Nile red-positive signal in animals with inhibited *alkb-8* (Fig. 6).

We also assayed whether forced expression of *alkb-8* affects the Nile red-positive fluorescence in enterocytes. Two transgenic lines expressing *alkb-8* from extrachromosomal arrays under the regulation of heat-shock-regulated promoter based on the plasmids pPD49.78 and

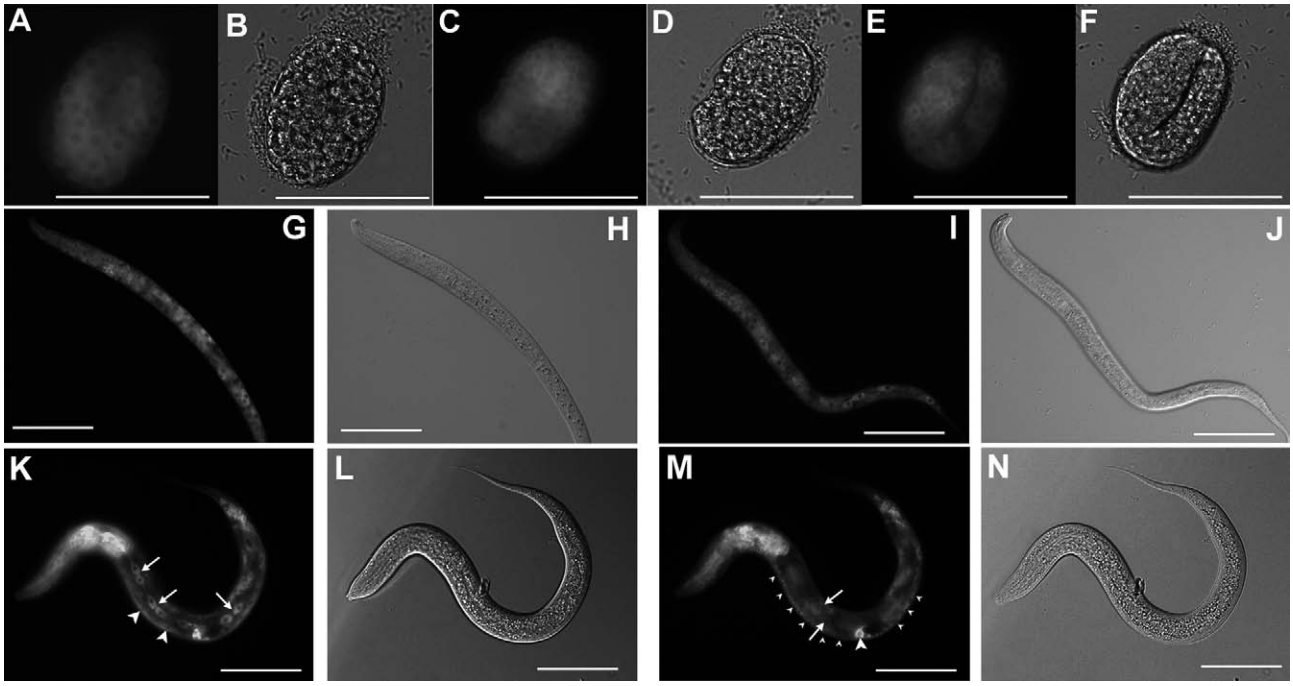

Fig. 3. Expression pattern of ALKB-8::GFP in early stages of development using a transgenic line carrying an extrachromosomal DNA construct

The construct composition is shown in Fig. 1. The GFP signal in embryos can be detected early after eggs are laid (around 40-cell stage) shown in panels **A** and **B**. The expression continues to be ubiquitous during embryonic development; panels **C** and **D** show an embryo at the end of the gastrulation phase, panels **E** and **F** an embryo at the 2-fold stage. Panels **G** to **J** show early L1 larvae, where the GFP signal is detected in all cell types with similar intensity. In the L1/L2 developmental stage (panels **K** through **N**), the expression starts to be differentiated and the highest signal is seen in pharyngeal and neuronal cells in the head and tail areas. Strong signal is also detected in seam (arrows) and muscle cells (arrowheads) in panel K. In panels **M** and **N**, the same animal is shown as in panels K and L, but with focus on a different layer. High expression is visible in intestinal cells (arrows), the distal tip cell (DTC) (arrowhead) and in the ventral nerve cord (small arrowheads). Pictures in panels A, C, E, G, I, K, and M are taken in GFP fluorescence and panels B, D, F, H, J, L, N in Nomarski optics. Bars represent 50 μm.

pPD49.83 were prepared. Both plasmids lead to the transgene expression in a wide spectrum of cells and differ in the extent of the expression in intestinal cells, which is higher in the case of pPD49.83. Both transgenic lines showed a strong decrease in the extent of Nile red-positive signal in enterocytes (Fig. 7). In keeping with the ALKB-8 intestinal role, the line based on pPD49.83, which leads to strong intestinal expression of the transgene, showed the lowest values for Nile red-dependent fluorescence.

The effect of alkb-8 overexpression on C. elegans lifespan

To determine whether the effect of ALKB-8 on the Nile red-positive compartment has a broader metabolic role, we assayed the lifespan of animals with downregulated *alkb-8* expression or pulse-overexpressed *alkb-8.* Downregulation of the *alkb-8* expression (applied for the entire lifetime of the assayed animals) had no effect on the animal lifespan (Fig. 8). In strong contrast, pulse-forced expression in animals during their L1 stage led to pronounced lifespan extension of experimental animals reaching 10 to 40 %.

Discussion

Our results support the ALKB-8 modulatory function in metabolic events linked to lysosome-related organelles and aging in *C. elegans*. Surprisingly, despite *alkb-8* being expressed strongly and ubiquitously from early embryonic stages to adulthood, its downregulation by RNAi to the levels that affect detection of lysosome-related organelles by *in vivo* Nile red staining do not harm embryonic development. This suggests that the sensitivity of lysosome-related organelles to ALKB-8 levels is greater than a possible involvement in developmental events. In keeping with the metabolic roles of ALKB-8, its overexpression applied during the first larval stage markedly prolonged the lifespan. On the other hand, downregulation of *alkb-8* by RNAi did not shorten their lifespan. There are several factors that may cause this discrepancy. Firstly, RNAi does not significantly affect neuronal cells in wild-type N2 *C. elegans*

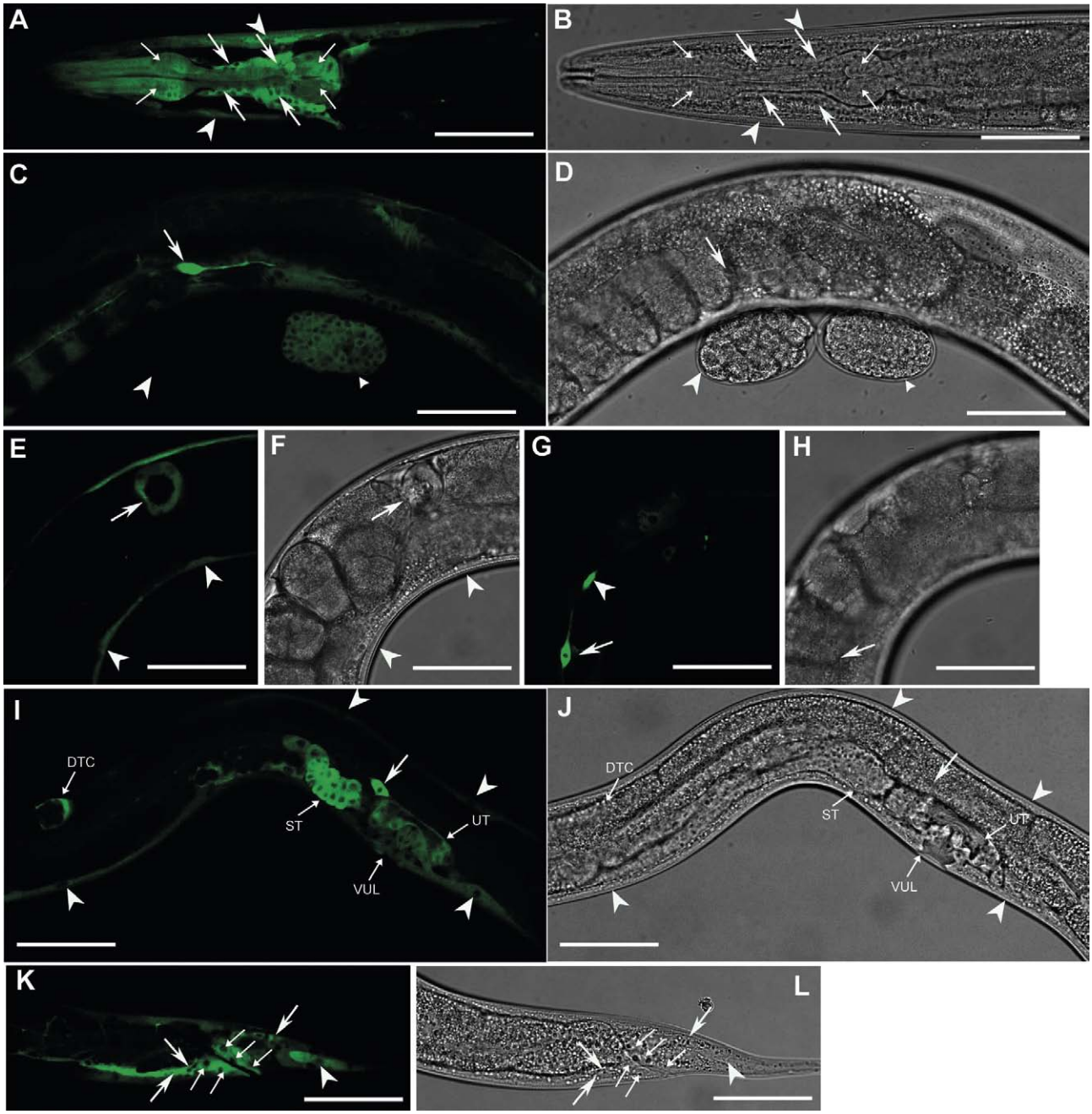

Fig. 4. Expression of *alkb-8::gfp* from extrachromosomal arrays regulated by the promoter of CEOP3136 operon analysed by confocal microscopy

Panel **A** shows the expression of *alkb-8::gfp* in the head of an adult animal. Strong signal is detected in neurons (arrows), pharyngeal muscle cells (small arrows) and head muscle cells (arrowheads). Panel **B** shows the same animal as panel A, but in Nomarski optics. Panels **C** and **D** show the central part of the body of an adult animal with two freshly laid embryos. The embryo on the left is approximately in the 30-cell stage (arrowhead) and shows no expression of *alkb-8::gfp.* In contrast, the embryo on the right is in the approximately 100-cell stage (small arrowhead) and shows ubiquitous cytoplasmic expression of the transgene. The canal-associated neuron (CAN) marked by an arrow shows strong cytoplasmic expression of the transgene. Panels **E** and **F** show expression of *alkb-8* in the spermatheca (arrow) and body wall muscles (arrowheads). Panels **G** and **H** show another focal plane of the same animal as showed in E and F. The arrow indicates strong expression in the CAN neuron and in another unidentified neuron (arrowhead). Panels **I** and **J** show the central part of the body of an L4 larva where high expression of *alkb-8* is detected in cells of the somatic gonad and egg-laying apparatus indicated by small arrows (DTC – distal tip cell, ST – spermatheca, UT – uterus, VUL – vulva). Large arrow points to the CAN neuron, arrowheads point to body wall muscles. Panels **K** and **L** show the distant part of an L4 larva with many *alkb-8*-positive cells. Tail neurons (arrows), hyp cell (arrowhead) and rectal epithelial cells (small arrows) are indicated. Panels B, D, F, H, J, and L show the same picture as the fluorescent picture on their left in Nomarski optics. Bars represent 50 μm.

unless specific lines are used for silencing experiments (Simmer et al., 2002), and thus a proportion of ALKB-8 responsible for the observed phenotypes may be unaffected in *alkb-8* downregulation experiments. The experiments with *alkb-8* forced overexpression are likely to lead to elevated levels of ALKB-8 in most cells, except in the gonads. It can be assumed that the effects on the extent of detection of the *in vivo* Nile red-positive compartment is at least partially a result of ALKB-8 direct function in enterocytes. The effect on longevity may to a large extent be based on the neuronal functions of ALKB-8. In agreement with this, in *rrf-3* mutant animals, in which RNAi also affects neuronal cells, neuronal inhibition of the autophagy nucleation complex extends the lifespan of *C. elegans*. The authors demonstrated that inhibition of the VPS-34/BEC-1/EPG-8 autophagic nucleation complex as well as its upstream regulators strongly extend the *C. elegans* lifespan and that post-reproductive inhibition of *bec-1* mediates longevity specifically through the neurons (Wilhelm et al., 2017).

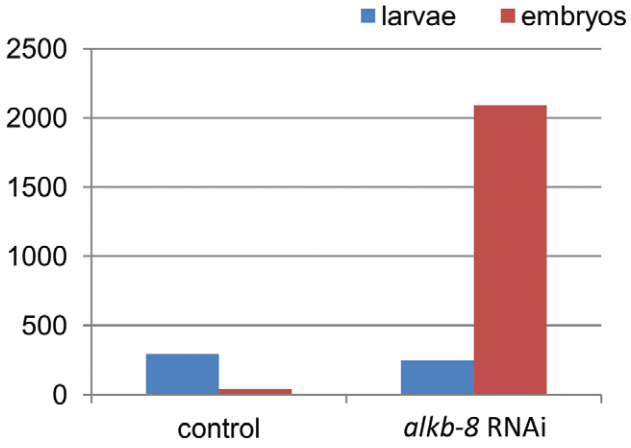

Fig. 5. Analysis of the effect of *alkb-8* downregulation by RNAi on *C. elegans* larval development
Equal amounts of synchronized L1 larvae were transferred to plates with control cultures (HT115 bacteria transformed with empty L4440 vector) and experimental plates seeded with bacteria transformed with L4440 vector containing *alkb-8* cDNA. Both control and experimental plates were induced using IPTG and the cultures observed to the time point when control animals started to lay eggs. At that time, equal areas of plates with nematodes were photographed and the number of animals (and laid eggs) was determined. The experiment shows that inhibition of *alkb-8* by the feeding method that was used in this experiment doesn't affect the larval development of *C. elegans*. In contrast, animals with downregulated *alkb-8* developed faster compared to control animals**.**

The positive effect of ALKB-8 on the lifespan may be connected with the short-term heat shock that was applied to both control and experimental animals in order to induce forced expression of the transgene. Nevertheless, the applied heat shock lasted only 30 min in the L1 larval stage, and the lifespan of control animals subjected to the short-term heat shock did not differ from the normal lifespan of animals kept under similar laboratory conditions but not subjected to the experimental heat shock. The involvement of ALKB-8 in other kinds of stress is supported by the known role of AlkB proteins in the stress response. The founding member of the protein family, the bacterial AlkB, is involved in the DNA damage-induced stress (Fedeles et al., 2015). ALKBH8 is known to regulate the rate of translation of thioredoxin reductase (Endres et al., 2015), which is one of the main enzymes important for dealing with oxidative stress (Li et al., 2012; Cunniff et al., 2014).

Our results as well as published data (Pastore et al., 2012) indicate the cytoplasm as the primary place of ALKB-8 action, although a low level of nuclear ALKB-8 cannot be ruled out. *alkb-8* is organized on chromosome III in hybrid operon CEOP3136. As such, it is trans-

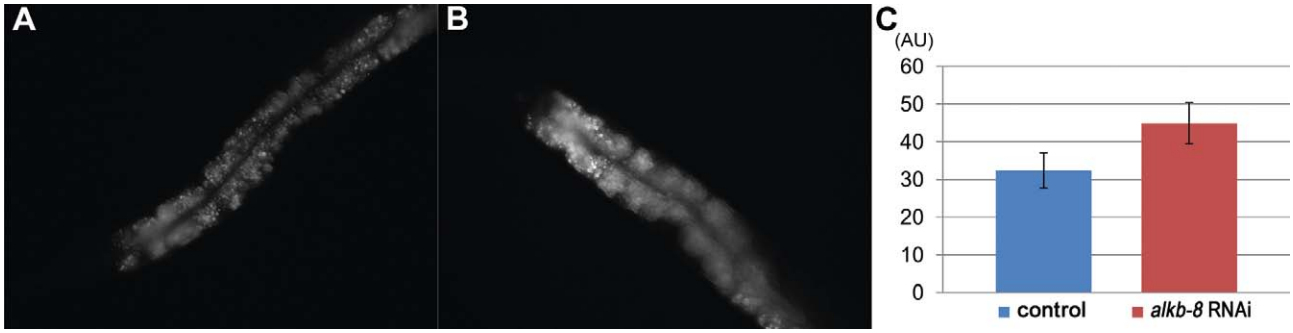

Fig. 6. Detection of the signal in the *in vivo* Nile red-stained compartment in control animals and animals with downregulated *alkb-8*
Panel **A** shows the Nile red-derived fluorescence in a young adult control animal. Panel **B** shows a larva with *alkb-8* inhibited by RNAi with the identical optical settings. Panel **C** shows the result of densitometric analysis of Nile red-derived fluorescence in the two most proximal enterocytes of 23 animals with downregulated *alkb-8* and 21 control animals. The results show a pronounced increase of approximately 30 % of Nile red-derived fluorescence in animals with *alkb-8* downregulated by RNAi compared to control animals. $P < 0.001$

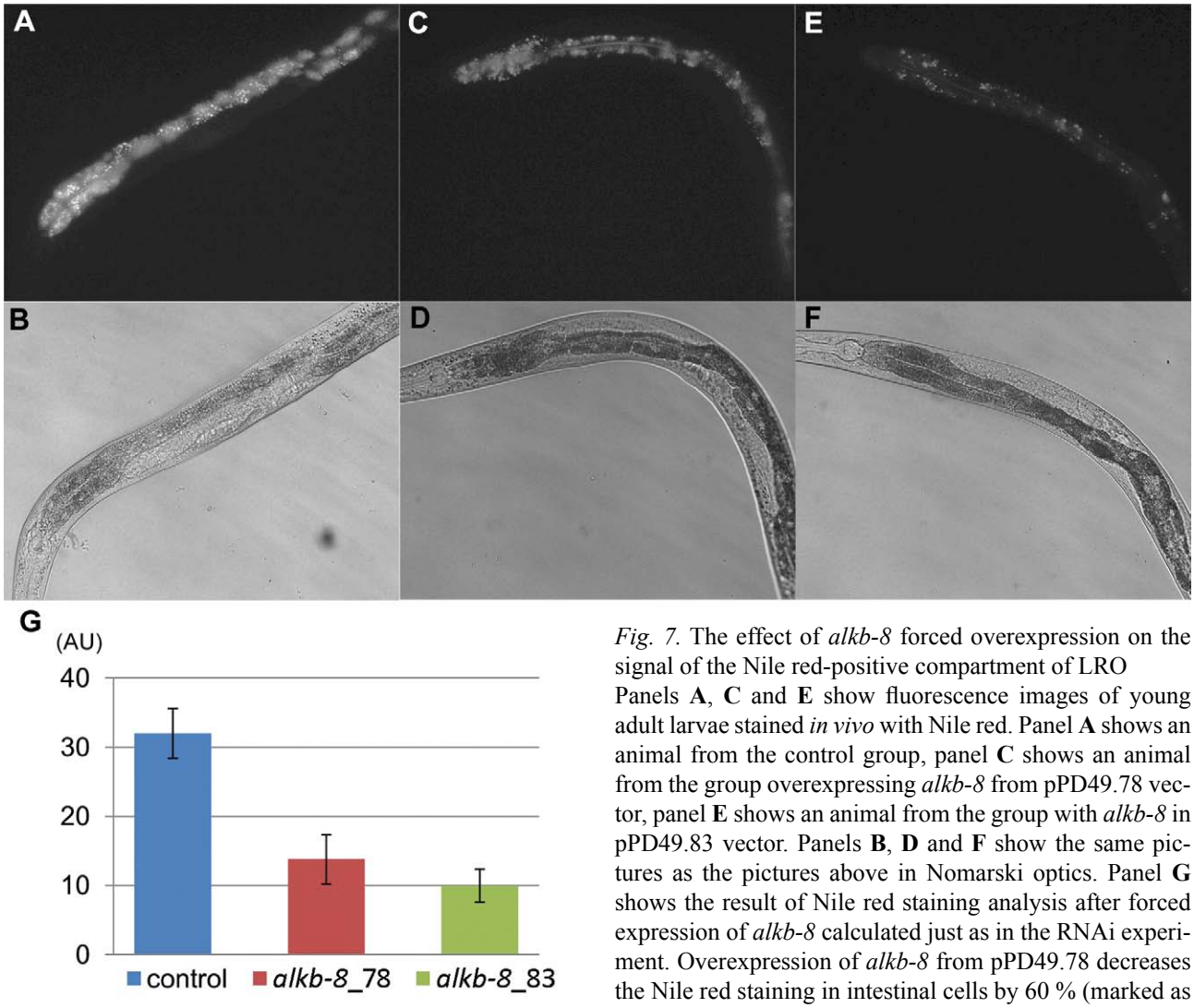

Fig. 7. The effect of *alkb-8* forced overexpression on the signal of the Nile red-positive compartment of LRO
Panels **A**, **C** and **E** show fluorescence images of young adult larvae stained *in vivo* with Nile red. Panel **A** shows an animal from the control group, panel **C** shows an animal from the group overexpressing *alkb-8* from pPD49.78 vector, panel **E** shows an animal from the group with *alkb-8* in pPD49.83 vector. Panels **B**, **D** and **F** show the same pictures as the pictures above in Nomarski optics. Panel **G** shows the result of Nile red staining analysis after forced expression of *alkb-8* calculated just as in the RNAi experiment. Overexpression of *alkb-8* from pPD49.78 decreases the Nile red staining in intestinal cells by 60 % (marked as *alkb-8*_78) and from pPD49.83 (marked as *alkb-8*_83) by 70 % compared to control animals. $P < 0.0001$

spliced with both SL1 and SL2 splice leaders, indicating that part of the expressed forms of *alkb-8* depend on the operon promoter and the other part on the internal *alkb-8* promoter. The expressional pattern of the transgene expressed under the regulation of the operon promoter (used in our study) is very similar if not identical with the data reported for the internal *alkb-8* promoter (Pastore et al., 2012). Our experiments as well as the data reported by WormBase (release WS263) (Byrne et al., 2007) detected *alkb-8* expression in intestinal cells. It is therefore likely that the effect of *alkb-8* inhibition and overexpression is at least partially caused by intestinal ALKB-8.

ALKB-8 (from amino acid position 362 to the end) shows significant homology to yeast methyltransferase TRM9 (TRM9_YEAST) not only in the SAM binding part, but also at the C-terminus. Deletion of TRM9 significantly increased the lifespan in *Saccharomyces cerevisiae* (Fabrizio et al., 2010), suggesting that ALKB-8 may act in the same pathway as the *C. elegans* orthologue of TRM9 (although in opposite ways). TRM9 is predicted to be important in protecting cells against protein stress (Patil et al., 2012). In *C. elegans* (and in most sequenced animal species), there is another gene that is similar to *alkb-8*, which only has the methyltransferase domain and not the demethylase domain C35D10.12 (NP_497751.1), but nothing is known about its function.

Our study shows that ALKB-8 regulates the function of the intracellular compartment that can be visualized by *in vivo* Nile red staining (Ashrafi et al., 2003) forming a distinct class of lysosome-related organelles (Soukas et al., 2013).

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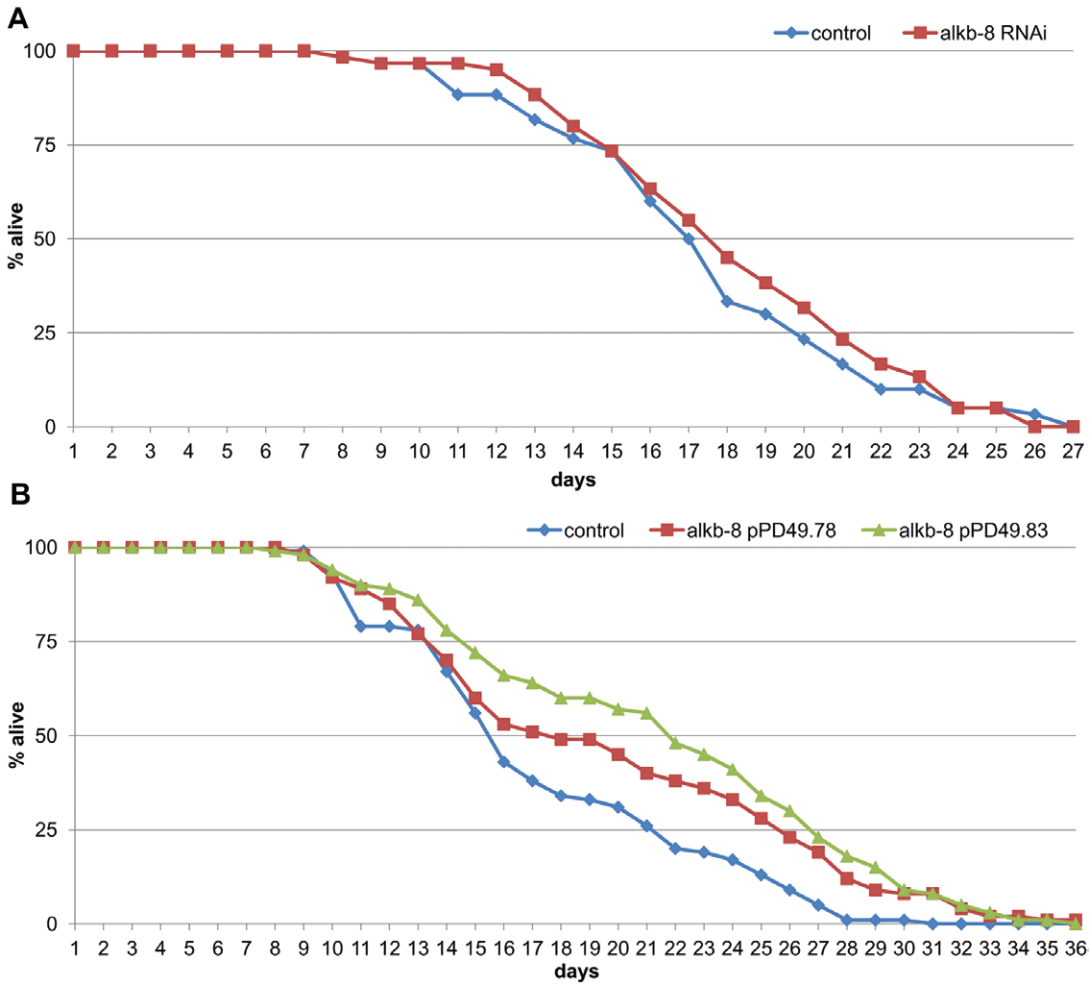

Fig. 8. Determination of the effect of *alkb-8* on the lifespan of *C. elegans*
Panel **A**. The effect of *alkb-8* downregulation on nematode longevity. Animals inhibited for *alkb-8* to the level affecting Nile red-positive compartment staining had no effect on the nematode longevity.
Panel **B**. The effect of pulse overexpression in L1 stage on *C. elegans* longevity. Compared to controls, animals with forced expression of *alkb-8* had the lifespan extended by 10 to 40 %.

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