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Regulation of gene expression at posttranscriptional levels

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Content:

ABBREVIATIONS ... 8

ABSTRACT ... 11

ABSTRAKT ... 12

HYPOTHESES ... 13

GOALS ... 13

1 INTRODUCTION ... 14

1.1 Regulation of gene expression ... 14

1.1.1 Regulation of gene transcription ... 14

1.1.2 Regulation on posttranscriptional levels ... 21

1.2 The model organism *Caenorhabditis elegans* as powerful tool for studies of gene expression regulation ... 28

1.3 ALKBH8 orthologues have a unique position in-between AlkB homologues 29

2 MATERIALS AND METHODS ... 33

2.1 Maintenance of *C. elegans* ... 33

2.1.1 Nematode cultivation ... 33

2.1.2 *C. elegans* strains ... 33

2.1.3 Synchronization of worm cultures ... 34

2.2 General molecular biology methods ... 34

2.2.1 Isolation of genomic DNA ... 34

2.2.2 Total RNA isolation ... 35

2.2.3 cDNA synthesis 35

2.2.4 PCR 36

2.2.5 Real-time PCR 36

2.3 Microscopy 36

2.4 Specific methods used for analysis of GEI-8 function in gene expression regulation 37

2.4.1 Microarrays 37

2.4.2 RNA interference 37

2.4.3 Staining with 4',6-diamidino-2-phenylindole (DAPI) 38

2.5 Methods used for characterization of ALKB-8 in *C. elegans* 38

2.5.1 RNA interference 38

2.5.2 Nile red staining 39

2.5.3 Overexpression of *alkb-8* 39

2.5.4 Preparation of *alkb-8*::*gfp* transgene regulated by CEOP3136 promoter and endogenous 3' UTR 40

2.5.5 Developmental assay 42

2.5.6 Life span determination 43

3 RESULTS 43

3.1 Whole genome expression analysis in animals without functional GEI-8 (CeNCOR) 43

3.1.1 Characterization of *gei-8* mutant strain 43

3.1.2 *gei-8* loss of function leads to transcription deregulation 45

3.1.3 21U-RNAs are involved in the defective development of gonad 46

3.2 Characterization of ALKB-8 in *C. elegans* 48

3.2.1 Analysis of the expression of *alkb-8* by RT-qPCR 48

3.2.2 Tissue- and cell-specific expression of *alkb-8* from the operon promoter 49

3.2.3 The effects of *alkb-8* downregulation on *C. elegans* development 52

3.2.4 The effect of *alkb-8* downregulation and forced overexpression on the visualization of the Nile red-positive compartment 54

3.2.5 The effect of *alkb-8* overexpression on *C. elegans* life span 56

4 DISCUSSION 58

4.1 GEI-8 loss of function leads to transcriptional deregulation 58

4.2 21U-RNAs of piRNA class are likely to be responsible for *gei-8(VC1213)* gonadal phenotype 59

4.3 ALKB-8 2-oxoglutarate and Fe^{2+} dependent dioxygenase and TRM-9 related methyltransferase regulates biology of Nile-red in vivo stained lysosome-related organelles (LRO) in *C. elegans* 61

4.4 ALKB-8 regulates lifespan in *C. elegans* 61

5 CONCLUSIONS AND EVALUATION OF GOALS AND HYPOTHESES .. 64

References 65

Supplementary files 75

ABBREVIATIONS

ADAR	Adenosine Deaminase, RNA Specific
AlkB	Alkylated DNA repair protein AlkB
ALKBH8	AlkB homologue 8
ALKB-8	AlkB homologue 8 in *C. elegans*
ama-1	AMAnitin resistant (*C. elegans* gene for large subunit of polymerase II)
ATP	Adenosine triphosphate
bp	base pair
CREB	cAMP response element binding
CTD	C-terminal domain
DNA	Deoxyribonucleic acid
dsRNA	double stranded RNA
DTC	distal tip cell
FTO	fat mass and obesity-associated protein
GEI-8	GEX Interacting protein (*C. elegans* NCOR/SMRT homologue)
GFP	Green fluorescent protein
GO	Gene Ontology
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
lncRNA	long non-coding RNA
LRO	lysosome related organelle
MED	Mediator

MDT	Mediator in *C. elegans*
mRNA	Messenger RNA
miRNA	Micro RNA
NCBI	National Center for Biotechnology Information
NCOR	Nuclear receptor co-repressor
NHR	Nuclear hormone receptor
NR	Nuclear receptor
P300	EP300 or E1A binding protein p300
PCR	Polymerase chain reaction
PIC	Preinitiation complex
piRNA	Piwi-interacting RNA
RT-qPCR	Reverse transcription quantitative PCR
RAR	Retinoic acid receptor
rRNA	ribosomal RNA
tRNA	transfer RNA
RNA	Ribonucleic acid
RNAi	RNA interference
RNA Pol II	DNA dependent RNA polymerase II
SANT domain	Switching-defective protein 3 (Swi3), Adaptor 2 (Ada2), Nuclear receptor co-repressor (N-CoR) and transcription factor (TF)IIIB
shRNA	small hairpin RNA
siRNA	Small interfering RNA
SMRT	Silencing mediator of retinoid and thyroid receptors

snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
snRNP	small nuclear ribonuclear protein
sqrd	Sulfide Quinone oxidoReDuctase
TAF	TATA-binding protein associated factor
TBP	TATA box-binding protein
TF	Transcription factor
TRM9	tRNA methyltransferase
TRX	Thioredoxin
TRXR	Thioredoxin reductase
tiRNA	transcription initiation RNAs
UTR	Untranslated region
VDR	Vitamin D receptor

ABSTRACT

Regulation of gene expression in response to cellular and organismal needs is essential for sustaining organisms' survival and successful competition in the evolution of life forms. This regulation is executed at multiple levels starting with regulation of gene transcription, followed by regulation at multiple posttranscriptional levels. In this thesis, I focused on posttranscriptional mechanisms that contribute to gene expression regulation in the model organism *Caenorhabditis elegans* which enables powerful genetic and genomic techniques and allows the visualization of experimental genetic manipulations *in toto*, on the level of the complete organism during its life span. For this, we analysed the function of the orthologue of mammalian transcriptional corepressor NCOR, GEI-8. We used a functionally defective mutant *gei-8(ok1671)*. I analysed the whole genome expression of homozygous *gei-8(ok1671)* mutant and its link with observed mutant phenotype that includes defective gonad development and sterility and performed experiments leading to the proposition that disbalances in 21-U RNAs of piRNA class present in the most derepressed gene, the predicted mitochondrial sulfide:quinine reductase encoded by Y9C9A.16, are associated with the gonadal phenotype. In the second part of the thesis, I focused on the function of an RNA modifying enzyme that is likely to fundamentally contribute to posttranscriptional modification of several classes of RNA, the nematode orthologue of ALKBH8, named ALKB-8. Both the nematode and vertebrate orthologues contain three functional domains, an N-terminal RNA binding motif, a 2-oxoglutarate-dependent dioxygenase module homologous to bacterial AlkB, which oxidatively demethylates DNA substrates and a methyltransferase domain homologous to yeast TRM9, which selectively modulates translation of mRNAs enriched with AGA and GAA codons under both normal and stress conditions. We show that downregulation of *alkb-8* increases the extent of lysosome-related organelles visualized by Nile red *in vivo* and reversely, forced expression of *alkb-8* strongly decreases the detection of this compartment. Overexpression of *alkb-8* applied in a pulse during the L1 larval stage projects to increased life span of *C. elegans*. Together, our results identified new regulatory pathways based on posttranscriptional mechanisms and contributed new data supporting the concept of extensive posttranscriptional mechanisms modulating gene expression to comply with organism's needs.

ABSTRAKT

Regulace genové exprese v odpovědi na potřeby buněk a organismů je zásadní pro přežití organismů a úspěšnou kompetici v evoluci forem života. Tato regulace je prováděna na mnohočetných úrovních počínaje genovou transkripcí, následovanou regulacemi na četných posttrankripčních úrovních. V této disertační práci jsem se zaměřila na posttrankripční mechanismy, které přispívají k regulaci genové exprese v modelovém organismu *Caenorhabditis elegans,* který umožňuje efektivní genetické a genomické přístupy a vizualizaci důsledků experimentálních manipulací *in toto*, na úrovni celého organismu v jeho kompletním životním cyklu. Pro dosažení tohoto cíle jsme analyzovali funkci proteinu GEI-8, ortologu transkripčního korepresoru NCOR. Použili jsme funkčně defektivní mutantu *gei-8(ok1671).* Analyzovala jsem celogenomovou expresi homozygotní mutanty *gei-8(ok1671)* a její vztah k pozorovanému fenotypu zahrnujícímu defektivní vývoj gonády a sterilitu. Provedli jsme experimenty, které podporují regulační zapojení 21U-RNA třídy piRNA exprimovaných z de-reprimovaného genu Y9C9A.16, kodujícího předpovězenou mitochondrialní sulfide:quinine reduktázu, ve vývoji gonadálního fenotypu. V druhé části disertační práce jsem se soustředila na funkci RNA modifikujícího enzymu ALKB-8, orthologního k ALKBH8 obratlovců, který je pravděpdobným modifikátorem několikerých tříd RNA. Tento enzym má zachovalý N-terminální motiv vážící RNA, 2-oxoglutarát a Fe^{2+} dependentní dioxogenázový modul homologní s bakteriální demethylázou AlkB a DNA methyltransferázový modul homologní ke kvasinkovému TRM9, který moduluje translaci mRNA molekul obsahujících AGA a GAA kodony v odpovědi na stresové podmínky. Ukázali jsme, že snížení exprese *alkb-8* zvyšuje rozsah lysosomálního kompartmentu charakterizovaného detekcí Nilskou červení v metodě *in vivo* a obráceně, nadměrně indukovaná exprese velmi silně snižuje rozsah tohoto kompartmentu. Nadměrná exprese *alkb-8* v jediné aplikaci během larválního vývoje vede k prodloužení délky života *C. elegans.* V souhrnu, naše výsledky identifikují nové regulační cesty založené na posttrankripčních mechanismech a podporují koncept extensivních posttranskripčních mechanismů modulujících genovou expresi v souladu s potřebami organismu.

HYPOTHESES

Despite that regulation of gene expression is tightly connected with organisms' needs, numerous causes are likely to lead to disbalances in overall gene expression on the mRNA and on the protein levels. Additional regulatory steps are needed to help individual cells, tissues and organisms to overcome such disbalances and sustain cellular homeostasis. We hypothesized that a detailed analysis of model situations in *Caenorhabditis elegans* may contribute to further understanding of regulation of gene expression in response to metabolic and developmental needs.

GOALS

Our goal was to search for regulatory mechanisms that can adjust gene expression at posttranscriptional levels in the model organism *Caenorhabditis elegans* on the level of the complete organism.

My specific experimental goal was to study the genome-wide expression in *gei-8(ok1671)* homozygous mutants and search for a connection between the observed expressional profile and phenotype of defective gonad development.

The second specific experimental goal was to perform a complete functional analysis of ALKB-8 and to identify processes in which ALKB-8 was expected to perform its function at posttranscriptional levels.

1 INTRODUCTION

1.1 Regulation of gene expression

A precise regulation of which gene is expressed at particular time is essential for proper cell function. Specific gene expression determines the cell fate in development and it determines cell behaviour as a part of an organism and in response to different intra- and extracellular signals. Probably each step in the process of protein synthesis from its gene may be regulated. Defects in gene expression regulation are often associated with a variety of diseases (Reinke et al.,2013).

1.1.1 Regulation of gene transcription

The first step in gene expression is transcription of gene sequence from DNA into RNA. Although known in detail, the current understanding of steps that result in activation of RNA synthesis by RNA polymerase II are undergoing dramatic development. RNA polymerase II that is responsible for transcription of protein coding genes (Hsin and Manley,2012; Hsin et al.,2014) and regulatory RNAs including miRNA(Lee et al.,2004), siRNA(Yuan et al.,2006), piRNA (Weick and Miska,2014) and lncRNA RNA classes (Wang and Chang,2011) is organized in the basal transcriptional machinery together with general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. This multiprotein complex upon activation including phosphorylation of its C-terminal domain executes transcription of the primary transcripts (the pre-mRNAs). One of the general transcription factors TFIID, itself a complex consisting of TATA box-binding protein (TBP) and 13 TBP associated proteins (TAFs), binds TATA box sequence in promoters of genes and contributes to activation of RNA synthesis from TATA-box containing genes (Duttke,2015; Juven-Gershon and Kadonaga,2010).

Transcription of most genes is however dependent on additional transcription factors, transcription activating proteins that bind to sequences present in promoters of cognate genes called response elements (Lemon and Tjian,2000). Approximately 2000 genes are recognized as transcription factors in human genome (Babu et al.,2004). Major contribution to our understanding of the mechanistic function of transcription factors in general was achieved in studies focused on proteins interacting with basal transcritptional machinery in yeast (reviewed in (Robinson et al.,2015), and SP1 and nuclear receptors in mammalian cells (reviewed in (Borggrefe and Yue,2011)). Independently, the work on liganded nuclear receptors (THRs, RARs and VDR) and SP1 transcription factor led to discoveries of major transcription activators that include protein paralogues CBP/P300 (CREB-binding protein or CREBBP, and EP300 or E1A binding protein p300, respectively) which possess an acetlytrasferase activity and acetylate lysines of nucleosomal histones in gene promoters (reviewed in (Dancy and Cole,2015)). This is generally connected with transcription activation. Opposed to CBP/P300, two major proteins NCoR1 and NCoR2 (a.k.a. SMRT) were identified as corepressors mediating transcription repression caused by unliganded nuclear receptors (such as THRs, RARs and VDR). NCOR1 and NCOR2 associate with and activate enzymes collectively classified as histone deacetylases (class I and II) (named HDACs). HDACs deacetylate acetylated lysines in promoters of regulated genes what is leading to transcription attenuation (Horlein et al.,1995; Chen and Evans,1995).

However, analyses of proteins found as interactants of transcription factors named above led to the realization that in both yeast and vertebrates, the signalling by transcription factors is transmitted to basal transcriptional machinery through a large multiprotein complex called Mediator complex or Mediator (Allen and Taatjes,2015; Amoasii et al.,2018; Bjorklund and Gustafsson,2004; Bjorklund and Gustafsson,2005;

Carlsten et al.,2013; Conaway et al.,2005; Fondell,2013; Grants et al.,2015; Jeronimo and Robert,2017; Poss et al.,2013; Soutourina,2018). Mediator consists of 20 to 25 subunits in yeast and more than 26 subunits in vertebrates (Fig. 1). Mediator complex subunits named MED (with the appropriate subunit number) in vertebrates and MDT (with the appropriate subunit number) in *C. elegans* form four distinct modules named Head, Middle, Tail and CDK modules (Allen and Taatjes,2015; Carlsten et al.,2013; Grants et al.,2015; Taatjes,2010; Yin and Wang,2014). The CDK module is dispensable for gene expression activation and has in most cases repressive role in Mediator complex mediated transcription (Carlsten et al.,2013).

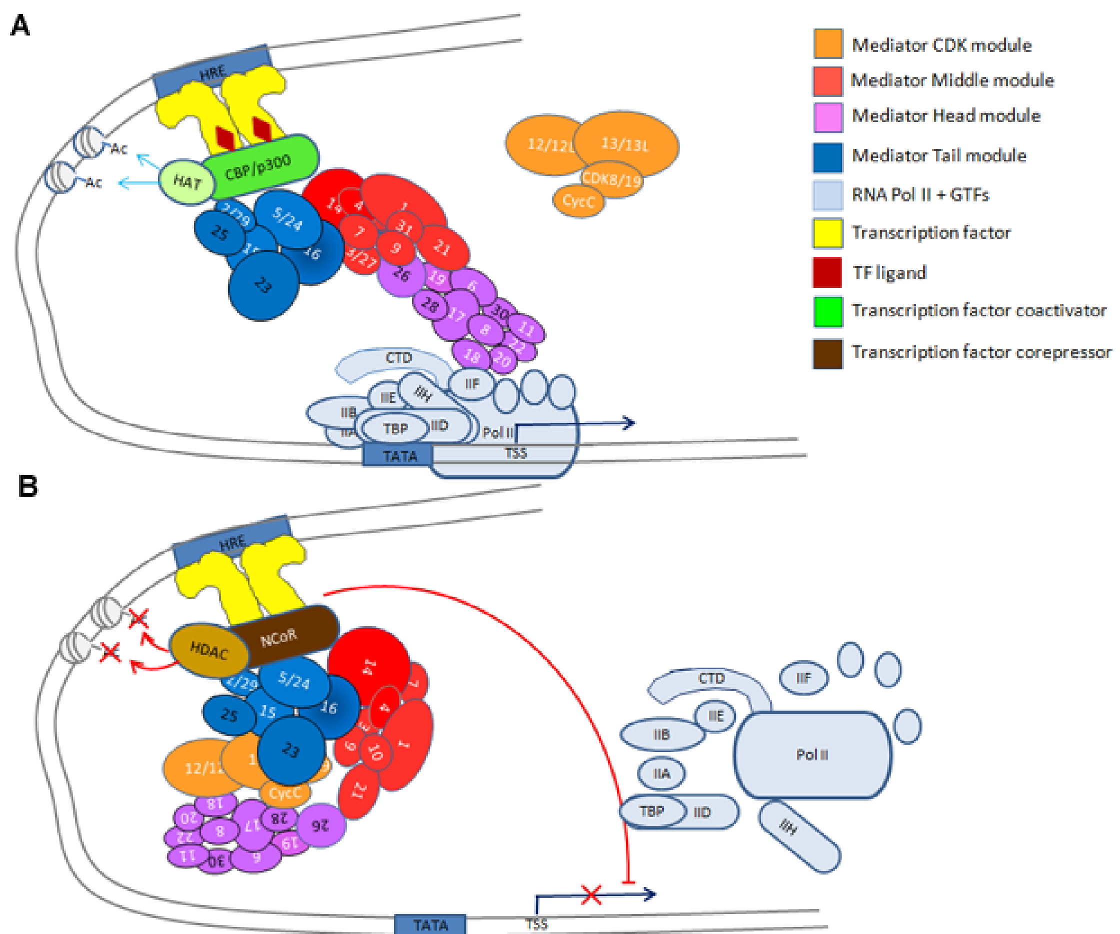

Fig. 1 Simplified scheme of regulation of gene transcription by nuclear receptors. Panel A – Schematic representation of transcription activation by a liganded nuclear receptor(s). Association of the activating ligand results in conformational change of the

ligand binding domain of nuclear receptors leading to exposition of protein surfaces allowing association with transcription co-activators CBP/p300 which possess intrinsic histone acetyl transferase activity and may also associate with additional transcriptional coactivators with histone acetyltransferase activity (such as NCOA1/SRC1). Acetyltransferase enzymatic activity leads to acetylation of nucleosomal histones in adjacent promoter of cognate genes which is further translated to elevated transcriptional activity of the particular regulated gene. Nuclear receptor is further transmitting its transcription activation potential towards basal transcriptional machinery through individual contacts with subunits of the Mediator complex which is further contacting basal transcriptional machinery. Panel B – shows repression of gene transcription by unliganded nuclear receptors. Unliganded nuclear receptors interact with transcription repressors NCOR1 or NCOR2 which bind histone deacetylases that deacetylate acetylated lysines found in vicinity in promoters of regulated genes. This translates to inhibition of gene transcription and release of basal transcriptional machinery from gene promoters. Mediator complex may be released or possibly acquire transcription repressive conformation with its generally transcription repressive CDK module (Carlsten et al.,2013; Conaway and Conaway,2011).

1.1.1.1 Additional roles of transcriptional correpressors orthologous to NCOR1

NCoR and SMRT are paralogous vertebrate proteins that were first identified as transcriptional corepressors interacting with unliganded nuclear receptors (thyroid and retinoid receptors) (Horlein et al.,1995; Chen and Evans,1995). Both NCoR (a.k.a. NCoR1, NCOR1) and SMRT (a.k.a. NCoR2, NCOR2) are big proteins coded by genes spanning about 200,000 bp.

NCOR1 is located on the chromosome 17 (NC_000017.11 (16030094..16216475, complement), spanning 186,381 bp and including more than 40 possible exons (https://www.ncbi.nlm.nih.gov/gene/9611, accessed on June 23, 2018). There are 45 different known *NCOR1* mRNA species. *NCOR2* is located on Chromosome 12 (NC_000012.12 (124324411..124567464, complement) and spans 243,053 bp, is similarly large gene as *NCOR1* and contains also at least 40 possible exons (https://www.ncbi.nlm.nih.gov/gene/9612, accessed on June 23, 2018). Three different species of mRNA of NCOR2 are known. The *C. elegans* orthologue *gei-8* is localized on chromosome III (III:8111166..8126222), spans 15,056 bp, includes 16

possible exons spliced to 6 known mRNA species. This suggests that many functions of individual protein species of mammalian NCORs as well as nematode GEI-8 are likely to be still unknown. The critical role of NCOR1 alternative splicing was proven by Martin Privalsky and co-workers in the regulation of adipocyte differentiation (Snyder et al.,2015).

Knockouts of *NCOR1* as well as *NCOR2* are embryonic lethal in mice suggesting that their regulatory roles are indispensable for normal embryonic development (Jepsen et al.,2000). NCORs function is connected with the assembly of a repressor complex composed of transcription factors, histone deactylases (HDACs), and other components (Goodson et al.,2005). This is followed by chromatin remodeling which depends on the formation of a stoichiometric complex between NCORs and HDACs (HDAC3) that is mediated by two SANT (a.k.a. MYB) domains located at the N terminus of NCORs. SANT domains are present in many nuclear receptor corepressors and related proteins and consist of three alpha-helices folded around a core formed by three hydrophobic amino acids (Boyer et al.,2004; Ogata et al.,1994; Tahirov et al.,2001). The N-terminus proximal SANT1 domain activates the HDAC3 deacetylase (Guenther et al.,2001; Yu et al.,2003) and is referred to as the deacetylase activation domain (DAD). A prominent feature of all DAD domains is the absolutely conserved lysine residue (K449 in human NCOR2) that promotes HDAC3 activation but not its binding to the complex. The second SANT domain, SANT2, binds unacetylated histone H4 and increases affinity of NCORs to HDAC3 (Boyer et al.,2004; Yu et al.,2003). The SANT2 domain of NCORs shows close structural similarity to SANT domains present in several protein families. In contrary, SANT1 domain is unique to NCORs and its orthologues (Codina et al.,2005). The SANT1 domain contains a characteristic irregular N-terminal helix that is important for forming an

additional surface hydrophobic groove that contributes to the interaction with HDAC3 which allows bioinformatic recognition of NCOR orthologues from other SANT domain-containing proteins.

NCOR homologues can be easily identified in different vertebrate species but not in protostomes like *Drosophila* or *C. elegans* despite that other components of the corepressor complex like NuRD and SIN3 are present in their genomes (Ahringer,2000; Choy et al.,2007). The NCOR nematode orthologue GEI-8 was originally identified as a GEX-3 binding protein based on yeast-two-hybrid assays (Tsuboi et al.,2002). Large-scale RNAi experiments did not report any loss-of function phenotypes. Two reports including the publication that is connected with this thesis brought data indicating that GEI-8 is the nematode orthologue of NCORs (Mikolas et al.,2013; Yamamoto et al.,2011). GEI-8 thus offers new possibilities for functional analyses of NCOR role in biology of nematode species.

1.1.1.2 Additional steps in gene expression regulation

Transcription by RNA pol II includes additional regulated steps including RNA pol II pausing, transcription termination and mRNA processing (splicing and polyadenylation) (Bentley,2014; Lenasi and Barboric,2013).

1.1.1.3 Regulation of gene expression by chromatin structure

Genetic material in Eukaryotes is compacted in macromolecular complex called chromatin. Basic unit of chromatin is nucleosome which consists of about 150 bp long DNA segment wrapped around histone protein core. Core histones form an octamer consisting of two molecules each of histones H2A, H2B, H3 and H4. Nucleosomes are assembled into long linear arrays in which each nucleosome is connected by 10–70 bp

of linker DNA, with the length varying between species and cell types (Woodcock and Ghosh,2010). The histone octamer has high affinity for DNA and this ensures that nucleosome assembly is a significant barrier for enzymes requiring DNA access, and the folding or compaction of nucleosomal arrays can lead to additional constraints on nuclear processes. Therefore methods capable of regulation the positioning and stability of nucleosomes and methods for disruption of nucleosomal array condensation are necessary for proper gene expression (Swygert and Peterson,2014). Surfaces of nucleosomal histones, especially their N-termini are accessible for enzymes mediating their posttranslational modifications. These modifications regulate chromatin structure by changing histone electrochemical surfaces and recruit additional enzymes collectively termed chromatin remodeling enzymes that are ATP-dependent and regulate re-position of nucleosomes in respect to DNA sequence (Bannister and Kouzarides,2011; Swygert and Peterson,2014). Histone modifications include phosphorylation, methylation, acetylation, and ubiquitination which are translated to transcription activation or repression by accessibility of transcription activating proteins and change the chromatin compaction to relaxed or condensed chromatin which has been predicted to facilitate or prohibit association with additional transcriptional regulatory complexes (Balakrishnan et al.,2010; Balakrishnan and Milavetz,2010).

Protein variants of histones, such as H2A.z or Histone H3 related protein CENPA are incorporated into the core histone octamer under the regulation of noncoding RNA in specific chromosomal regions including centromeric regions (Stimpson et al.,2010; Stimpson and Sullivan,2010).

1.1.2 Regulation on posttranscriptional levels

Following the transcriptional initiation till the decoding of codons written in mRNA sequence into amino acid sequence of protein on ribosomes most of the regulation of gene expression occurs through the action of RNA binding proteins and processing factors that associate with RNAs (Corbett,2018). All main species of RNA molecules after they are transcribed undergo different processing steps and modifications to fulfil their specific roles in the process of gene expression

1.1.2.1 mRNA processing

The precursor mRNAs undergoes extensive post-transcriptional processing. The first mRNA processing step includes 5' capping, during which a guanine nucleotide is connected to 5' triphosphate link and is methylated on the position 7 by methyltransferases structurally similar Rossmann Fold Methyltransferase superfamily. Capping of 5' end of mRNA makes both ends of mRNA structurally similar and has multiple roles in gene expression, including enhancement of mRNA stability, splicing, nucleocytoplasmic transport, and translation initiation (Byszewska et al.,2014). The second processing step that is linked with pre-mRNA transcription is its splicing which eliminates intronic sequences and is responsible for formation of multiple species of mRNAs originating from a single gene and which is responsible for formation of multiple species of final translated proteins coded by the same gene (Le et al.,2015). Following the transcription termination, mRNA is polyadenylated on its 3' end (Hollerer et al.,2014; Neve et al.,2017). This step is recently unfolding as complex mechanism dependent on cis elements present in pre-mRNA and multiple proteins representing trans-factors working in accord and linking 5' capping and splicing of pre-mRNA. The 3′end processing is dependent on the assembly of the multiprotein processing complex

on the pA signals that reside in the pre-mRNAs. Most eukaryotic genes have multiple pA signals what is a basis for alternative pre-mRNA cleavage and polyadenylation (APA) what in turn leads to formation of cell state and cell type specific transcriptomes and finally proteomes (Neve et al.,2017).

1.1.2.2 RNA editing

RNA editing is a process which results in sequence variation in the RNA molecule, and is catalyzed by enzymes. RNA editing was originally found in *Trypanosoma* where the mRNA of the mitochondrial gene coding for cox-2 was found to differ from its expected sequence in terms of four missing uridines at the 5′ end in comparison to the respective DNA sequence (Benne,1989; Benne et al.,1986). In higher eukaryotes, RNA editing was found in two types of hydrolytic deamination reactions: deamination of cytidine leading to the conversion to uridine (C-to-U), which is dependent on the APOBEC ("apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like") gene family and auxiliary proteins (Blanc and Davidson,2010). The second type of enzymatic editing is deamination of adenosine leading to formation of inosine which is dependent on the ADAR ("adenosine deaminase acting on RNA") enzyme gene family (Walkley and Li,2017; Wang et al.,2013).

1.1.2.3 RNA nucleotide modification

Posttranscriptional chemical modification of ribonucleotides is another way how to affect stability and function of RNA molecules and thus affect the gene expression on posttranscriptional level. Presently 109 different posttranscriptional modifications of RNA nucleotides are known. These modifications are found in all three major RNA species, ribosomal RNA (rRNA), messenger RNA (mRNA) and transfer RNA (tRNA), as well as in some other RNA species such as micro RNA (miRNA), small nuclear

RNA (snRNA), small nucleolar RNA (snoRNA) etc., and in all three phylogenetic domains, Archaea, Bacteria, and Eucarya (Cantara et al.,2011). How nucleotide modifications affect all main RNA species is briefly described below.

mRNA

The most prevalent modification in mRNA is N^6-methyladenosine (m^6A). Its presence in mRNA was discovered in1970s but its function remain mysterious for a long time. Only recent discovery of protein "writers", "erasers" and "readers" of this RNA chemical mark contribute to understanding of its function and point out that the mRNA modifications are dynamic, reversible and regulated (reviewed in (Fu et al.,2014; Nachtergaele and He,2017)). First were discovered demethylases of m^6A: FTO (Jia et al.,2011) and ALKBH5 (Zheng et al.,2013), both belonging to the AlkB family of 2-oxoglutarate and Fe(II) dependent dioxygenases. Methylation of m^6A mRNA is catalysed by METTL3/METTL14 methyltransferase complex (Liu et al.,2014). The noticeable phenotypes of *FTO* and *Alkbh5* mutations in humans and mice strongly indicate the functonal importance of this reversible m^6A methylation on RNA. m^6A function is executed through binding of specific proteins. Protein YTHDF2 preferentially recognizes m^6A-containing mRNA and regulates both mRNA stability and localization (Fu et al.,2014). Other modification as N^1-methyladenosine (m^1A), 5-methylcytosine (m^5C), pseudouridine (Ψ) and 2′-O-methylation (Nm) were also detected in mRNA but their functions remain to be elucidated (Nachtergaele and He,2017).

rRNA

Modifications of rRNA nucleotides serve to fine tune ribosome structure and function. The most prevalent modifications of rRNA nucleotides are pseudouridine (Ψ) and ribose 2′-O-methylation (Nm), but other modifications were also identified in eucaryotic rRNA (for example N^1-methyladenosine (m^1A), N^6,N^6-dimethyladenine ($m^{6,6}A$), 5-methylcytosine (m^5C), N^4-acetylcytidine (ac^4C), N^7-methylguanosine (m^7G)). The modifications cluster in regions of the ribosome that have functional importance, a high level of nucleotide conservation, and typically lack proteins. A wide spectrum of chemical diversity from the modifications provides the ribosome with a broader range of possible interactions between ribosomal RNA regions, transfer RNA, messenger RNA, proteins, or ligands by influencing local ribosomal RNA folds and fine-tuning the translational process (Chow et al.,2007; Nachtergaele and He,2017).

An interesting association of changed rRNA modification status with changes in translational control during tumorigenesis was reported by Jean-Jacques Diaz and collegues in 2015 (Marcel et al.,2015). These authors found that alteration in rRNA 2′-O-methylation pattern induced by changes in expression of fibrillarin (*FBL*), a conserved rRNA methyl-transferase guided by C/D box small nucleolar RNAs (snoRNAs), promotes the translation of a subset of mRNAs encoding proteins with oncogenic properties, which favors tumor initiation and progression (Marcel et al.,2015).

tRNA

The most heavily modified RNA species is tRNA, where over 80 different modifications were found (Grosjean,2005). All cells devote a great deal of resources to the tRNA modification. In fact, 1% to 10% of genes in a given genome encode enzymes

involved in tRNA modification. It is a proportion greater than the amount of genetic information devoted to encoding all the tRNA genes. This highlights the importance of modifications at all levels of tRNA function (El Yacoubi et al.,2012).

Modifications in tRNA may be roughly divided into two groups according to their predominant function in either structural and metabolic stabilization or in mRNA decoding. Many modifications within the structural core of the tRNA are essential to stabilizing the overall structure of the tRNA, and the loss of these modifications can result in rapid degradation of hypomodified tRNAs. Other modifications, especially those localized in the anticodon loop region, affect the function of the tRNA. These modifications contributes to the efficiency and accuracy of translation, e.g. by improving the recognition of tRNA by elongation factors or aminoacyl-tRNA synthetases, influencing codon-anticodon interaction and preventing frameshifting (Manickam et al.,2015; Nachtergaele and He,2017; Urbonavičius et al.,2001). Function of wobble uridine modification introduced by enzyme ALKBH8 (ALKB-8 in *C. elegans*) is described in more detail in chapter ALKBH8 orthologues have a unique position in-between AlkB homologues.

1.1.2.4 Regulatory RNAs

Regulatory RNAs gained a central role in regulation of gene expression over last 3 decades. They were shown to operate at multiple levels, from the regulation of transcribed mRNA to epigenetic processes that control cell differentiation and development of organisms (Morris and Mattick,2014).

There is an increasingly growing list of regulatory RNAs that were discovered almost in parallel and which discoveries helped mutually to uncover the mechanism of their functions.

Micro RNAs

Micro RNAs (miRNAs) were first discovered in *C. elegans* by Ambros and colleagues who showed that small genetic loci *lin-4* and *lin-7* regulate developmental timing (called collectively heterochronic genes) (Lee et al.,1993; Reinhart et al.,2000). It has been immediately recognized that the mechanism that employs these regulatory sequences is evolutionarily conserved and the regulatory sequences are also surprisingly well conserved from nematodes to humans (Pasquinelli et al.,2000).

RNA interference (RNAi)

The following step was the discovery of posttranscriptional gene silencing by double stranded RNA by Andrew Fire, Craig Mello and coworkers in *C. elegans*. This mechanism is referred to as RNA interference or RNAi (Fire et al.,1998). These authors also laid basis for the mechanistic analysis of the silencing mechanisms by exogenous double-stranded RNAs, their processing into short interfering RNA fragments and connection to miRNAs, suggesting a common endogenous system and finally to elucidation of the whole regulatory cascade (reviewed in (Morris and Mattick,2014)).

siRNA

The following class of regulatory RNAi was suggested by Thomas Tuschl and coworkers who showed that exogenous double-stranded 21-nucleotide long RNAs mediate RNA interference in cultured mammalian cells without the earlier observed stress response caused by long dsRNAs (Elbashir et al.,2001). These exogenous short double-stranded RNAs retained the classification as siRNAs.

Although there are several mechanisms that may lead to the formation of short double stranded RNAs in all organisms, the most pronounced mechanism of their formation is based on the expression of short RNAs that are partially complemental in

their sequence what leads to the formation of short hairpin RNAs, **shRNAs.** Mechanistically, shRNA are related to RNAi in formation of double-stranded RNA that is processed by the RNAi mechanism and results in formation of short dsRNA cleaved on both ends and forming short double-stranded RNA fragments not distinguishable from those used in siRNA. This mechanism is found to take place more widely in plants than animals. It is however very promising as an experimental and therapeutic tool (reviewed in (Morris and Mattick,2014).

piRNAs

The effort to elucidate the mechanism of RNAi led to discovery of individual protein and RNA constituents of the pathway including the Argonaute proteins (Ago clade proteins) and based on sequence similarity Piwi proteins (P-element induced wimpy testis) first identified as factors involved in germline stem cell (GSC) maintenance in *Drosophila melanogaster.* A new class of short noncoding RNAs was then identified as RNAs associated with Piwi proteins and their phenotypes related to regulation of germline development and silencing of mobile and repetitive sequences. Piwi RNAs are found in large numbers reaching thousands in mouse, human, *Drosophila* and *C. elegans* genomes (reviewed in (Morris and Mattick,2014).

In *C. elegans* they were identified in the earliest stages of piRNA centered research as sequences scattered though the genome and classified as 21U-RNAs (21 nucleotides long RNAs ending on their 5’ end with uridine) and similar, slightly different sequences such as 22G-RNAs. 21U-RNAs are formed by the endonuclease Zucchini from longer stretches of primary transcripts with little sequence specificity but a bias for leaving uridines at their 5’ ends.

The work connected with this thesis is linked to piRNAs in several aspects. In the time, when our primary report was published (Mikolas et al.,2013), it was not clear

how 21U-RNAs are formed. Our data thus provided direct support for their biogenesis dependency on transcriptional co-repressor NCOR/GEI-8 and Polymerase II (more can be found in the discussion).

Other classes of small RNAs in eukaryotes

The list of regulatory RNAs is extending to newly identified RNAs species including transcription initiation RNAs (tiRNAs), splicing regulating RNAs (spliRNAs) and long noncoding RNAs (lncRNAs). Another forms of small RNAs are formed from fragments of tRNAs in tissue-specific patterns and association with Ago proteins (tRNA-derived small RNAs) . This mechanism is still not well understood (reviewed in (Morris and Mattick,2014).

1.2 The model organism *Caenorhabditis elegans* as powerful tool for studies of gene expression regulation

C. elegans is currently established as a major model organism in biology. Many of the general characteristics make *C. elegans* an excellent model system for developmental studies and for precise and detailed analysis of gene expression regulation. This is further strengthen by the small size of all life stages of *C. elegans*, their microscopic transparency and short life cycle, altogether allowing to monitor consequences of experimental manipulations during the entire life of these animals. The model system offers a large width of very powerful genetic and genomic methods. The system allows comparisons of individually obtaied experimetnal data with a fast growing knowledge gained by the whole scientific community and shared through the bioinformatic tool named WormBase.

This allows to view experimental data now including the knowledge of the complete genome with its protein coding and regulatory sequences, many types of

regulatory RNAs, expressional data for individual life stages and individual cell and tissue types as well as collection of genetic mutants with their functional description and functional data of knock-down experimetns covering almost the whole genome and predicted as well as confirmed transcripts.

The model organism *C. elegans* is thus a very suitable system for analyses of gene expression regulation at tle level of gene transcription as well as additional downstream levels including regulation by regulatory RNAs (Reinke et al.,2013).

This model is used in our laboratory for studies on gene expression regulation by transcription factors from the superfamily of nuclear receptors and for elucidation of their mechanism of function (Kostrouchova and Kostrouch,2015). In this thesis, the model system of *C. elegans* was used for studies aimed at visualization of regulatory mechanisms likely to participate on gene expression regulation at additional posttranscriptional levels.

1.3 ALKBH8 orthologues have a unique position in-between AlkB homologues

The 2OG/Fe(II) (2-oxoglutarate- and Fe^{2+}-dependent) oxygenase superfamily possess an important position in-between oxygenases. The heme group is substituted in these enzymes by a protein module that coordinates Fe^{2+} and whose enzymatic activity is dependent on 2-oxoglutarate that serves as an electron donor and is consumed during the enzymatic reaction while converted to succinate and carbon dioxide. Unlike monooxygenases that are dependent on heme and which transfer one oxygen atom to the substrate and reduce the other oxygen 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 enzyme catalytic activity for 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 the regulation of hypoxia responsive genes (Aravind and Koonin,2001; Fedeles et al.,2015; van den Born et al.,2011).

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 9 genes, named ALKBH1 to 8 and a fat mass and obesity associated protein FTO originally identified as a gene localized at a chromosomal locus associated with the rat fussed-toes phenotype (Fedeles et al.,2015; Gerken et al.,2007; Peters et al.,1999). *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 a homeobox gene IRX3 that 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 lead 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 methyl transferase domain and an N-terminal RNA recognition motif that likely helps the AlkB domain in search for specifically modified tRNAs (Pastore et al.,2012; Songe-Moller et al.,2010). 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. It is regulated through the modification of bases in the anti-codon region of tRNA especially the wobble base, the first base in the anti-codon place of tRNAs, that can following this modification 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/gammaH2AX-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. showed that the AlkB domain of mammalian ALKBH8 catalyzes hydroxylation of 5-methoxycarbonylmethyluridine at the wobble position of tRNA (Fu et al.,2010a; Fu et al.,2010b). 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 a yeast methyltransferase TRM9. The function of the yeast TRM9 has been investigated in (Deng et al.,2015; Kalhor and Clarke,2003). The enzyme catalyses the 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 (Gu et al.,2014; Chan et al.,2010; Chan et al.,2012). 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 life span regulation and oxidative stress response in *Caenorhabditis 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 mitochondrial and protects mitochondria from oxidative stress, where reactive oxidative species are mainly generated, while cytosolic TrxR plays a role to maintain optimal oxido-reductive status in the cytosol. The cytosolic *trxr-1* is highly expressed in pharynx, vulva and intestine. *trxr-2* is mainly expressed in pharyngeal and body wall muscles and its defects cause a shortened life span and a delay in development under stress conditions. Deletion mutation of the 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 selenocystein-containing wild type TRXR-1, but also by a cysteine-substituted mutant TRXR-1. Both *trxr-1* and *trxr-2* were up-regulated 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 analyzed by electron microscopy, display

various level of birefringence in light microscopy and autoflorescence, 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).

2 MATERIALS AND METHODS

2.1 Maintenance of *C. elegans*

2.1.1 Nematode cultivation

In our laboratory the *C. elegans* cultures are maintaned as described (Brenner,1974). Animals are kept on Petri dishes with nematode growth medium (NGM) agar and fed with specific *E. coli* strains (OP50- used as natural food for *C. elegans* or HT115- used in RNAi experiments). Animals are visualized and manipulated using a dissecting microscope Olympus SD30 (Olympus, Tokyo, Japan)

2.1.2 *C. elegans* strains

As wild-type strain was used N2 (var. Bristol) strain obtained from the *C. elegans* Stock Center (https://cgc.umn.edu/).

The mutant strain VC1213 harboring a *gei-8*(*ok1671*) deletion allele was kindly provided by the *C. elegans* Gene Knockout Consortium. Prior to experiments, the mutant strain was backcrossed three times with wild-type males. The *gei-8* deletion

allele in mutant animals was confirmed by sequencing and the strain maintained as a heterozygote by scoring progeny mutant phenotypes.

Transgenic lines were prepared by microinjections of plasmid DNA into the ovarial syncitium of young adult N2 hermaphrodites using an Olympus IX70 microscope equipped with Narishige microinjection system (Olympus, Tokyo, Japan). Injections were kindly performed by Hana Prouzová (Prague, BIOCEV) as described (Fire et al.,1998; Tabara et al.,1999). Progeny of injected animals was screened for roller phenotype in case of ALKB-8 overexpression experiments or for GFP signal detected with Olympus SZX12 strereomicroscope System (Olympus, Tokyo, Japan) in case of ALKB-8::GFP expression experiment.

2.1.3 Synchronization of worm cultures

Synchronized populations of L1 larvae were prepared using the Bleching technique. In this technique worm cultures with high proportion of gravid hermaphrodites are treated with alkaline hypochlorite solution which destroys all larval stages except the embryos that are protected by egg shells. Embryos are then kept in PBS solution, where they can hatch but their further development is prevented without access to food. The protocol is described in (Porta-de-la-Riva et al.,2012).

2.2 General molecular biology methods

2.2.1 Isolation of genomic DNA

Genomic DNA from N2 wild-type animals was used as a template for some PCR reactions. For isolation we used High Pure PCR Template Preparation Kit (Roche

Diagnostics, Mannheim, Germany). DNA was isolated from approximately 50 mg of wild-type animals of mixed developmental stages. Worms were washed and pelleted by centrifugation, water was removed and then the manufacture´s protocol for “Isolation of Nucleic Acids from Mammalian Tissue” was followed.

2.2.2 Total RNA isolation

Total RNA was isolated from N2 animals of required developmental stage and feeding status. Animals, grown on 2% agarose plates, 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 60 min at 55 °C. RNA was isolated by phenol-chloroform extraction and ethanol precipitation and the pellet was dissolved in DEPC water. The sample was then treated with 1 unit of DNase I (New England Biolabs, Ipswich, MA) per 1µg of total RNA for 30 min at 37 °C. RNA was purified again by phenol-chloroform extraction and ethanol precipitation and pellet was dissolved in DEPC water.

2.2.3 cDNA synthesis

Complementary DNA (cDNA) used as template for PCR and quantitative PCR reactions was prepared by reverse transcription using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). We used about 1 µg of purified total RNA for first strand cDNA synthesis with use of random hexamer primers and we followed manufacturer’s protocol.

2.2.4 PCR

PCR reactions were performed according to a standard protocol using specific primers and templates (*C. elegans* gDNA or cDNA). For short fragments up to 1800 bp we used BIO-X-ACT™ Short DNA Polymerase (Bioline, London, UK) and for larger fragments we used AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). Reactions were run on PTC-200™ Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA).

2.2.5 Real-time PCR

Quantitative PCR (qPCR) was performed using the Universal Probe Library technique (Roche Molecular Systems, Inc. Pleasanton, CA, USA). Primers and probes were designed with Universal Probe Library System Assay Design Software. Reactions were run on LightCycler 2.0 with the software LightCycler 4.1 (Roche Molecular Systems, Inc. Pleasanton, CA, USA) and the protocol described in (Vohanka et al.,2010) was used. The expression was normalized against *ama-1* a large subunit of RNA polymerase II. All samples were run in triplicates.

2.3 Microscopy

Nomarski optics microscopy and fluorescence microscopy pictures were taken with Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). 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 Leica Application Suite X program (Leica Micosystems, Wetzlar, Germany).

2.4 Specific methods used for analysis of GEI-8 function in gene expression regulation

2.4.1 Microarrays

C. elegans whole genome expression microarrays (Affymetrix, Santa Clara, CA) were used to profile gene expression in three independent replicates based on manually selected homozygous *gei-8(ok1671)* mutants and matched N2 wild-type larvae at the earliest stage when mutants can be easily recognized based on their movement phenotype (L3 stage). Microarray chip data was analyzed by Affymetrix MAS 5.0 suite software (1.6-fold change in mRNA expression) and Robust Multichip Average (RMA) (1.2-fold change in mRNA expression) as part of the Partek genomics suite software package, all with a p-value less than or equal to 0.05. The microarray data has been deposited in the NCBI's GEO database (http://www.ncbi.nlm.nih.gov/geo) accession number GSE40127.

2.4.2 RNA interference

Y9C9A.16 dsRNA was synthesized using a 774 bp region of gDNA containing exons 2 to 6 amplified by primers 7501 and 7502 and cloned into pCRII vector (Invitrogen). Prior to in-vitro transcription by T7 or Sp6 polymerases, the construct was linearized. dsRNA was prepared by incubating ssRNAs at 70 °C for 10 min and at 37 °C for 30 min, followed by phenol-chloroform purification, ethanol-precipitation and dilution in DEPC water. dsRNA was injected into gonads of N2 wild-type hermaphrodites, heterozygous *gei-8(ok1671)* mutants, and homozygous wild-type

progeny of heterozygous *gei-8(ok1671)* mutant parents. *sqrd-1* RNAi was prepared using primers 7605 and 7606.

2.4.3 Staining with 4',6-diamidino-2-phenylindole (DAPI)

L4 stage homozygous *gei-8(ok1671)* mutants and N2 control animals selected from progeny of injected mothers were put on slides coated with poly-L-lysine and 20 µl of water, covered by the cover glass and put on dry ice. Freeze crack was performed after freezing the sample on dry ice for 5 minutes. Samples were kept in -20 ºC methanol for 10 minutes, then stained with DAPI (20µl, 1:1000 dilution of 1mg/ml) and mounted with fluorescent mounting medium (DakoCytomation, Copenhagen, Denmark) and coated with nail polish.

2.5 Methods used for characterization of ALKB-8 in *C. elegans*

2.5.1 RNA interference

For downregulation of *alkb-8* expression we used the RNAi feeding method where animals are fed on bacteria producing dsRNA as previously described (Timmons et al.,2001).

For preparation of 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, Carlsbad, CA, USA). Primers used for the PCR reaction were 11/08 and 10/08 (primer sequences are listed in Table 1.). Then the *alkb-8* sequence was recloned 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 from

each was inoculated to LB medium with Ampicillin (100 µg/ml final concentration) and let grown to $OD_{600} \approx 0,4$. Then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to culture to a final concentration of 0,4 mM to induce dsRNA production. The culture was grown for 4 hours 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.

2.5.2 Nile red staining

For the estimation of 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. 300 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 20x objective, exposure time 10 ms in RNAi experiments and 50ms in overexpression experiments). Resulting images were analyzed 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.

2.5.3 Overexpression of ***alkb-8***

The entire cDNA sequence of *alkb-8* was recloned from pCRII vector into the expression vectors which contain heat shock inducible promoter pPD49.78 and pPD49.83 using restriction enzymes EcoRV and KpnI. Constructs were injected into N2

hermaphrodites (at a concentration of 50ng/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 control we used animals injected only with pRF4 plasmid.

Forced expression was induced in a synchronized population of L1 animals. Larvae were placed on plates seeded with OP50 bacterial culture and were left for 2 hours 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 life span was determined. In case of Nile red staining experiment, the bacterial culture was supplemented with 15 ng of Nile red per plate. Pictures were taken after 50 hours using a constant setting.

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

According to WormBase (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 transgene expressing ALKB-8 tagged with 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, Carlsbad, CA, USA) according to the manufacture´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. 2

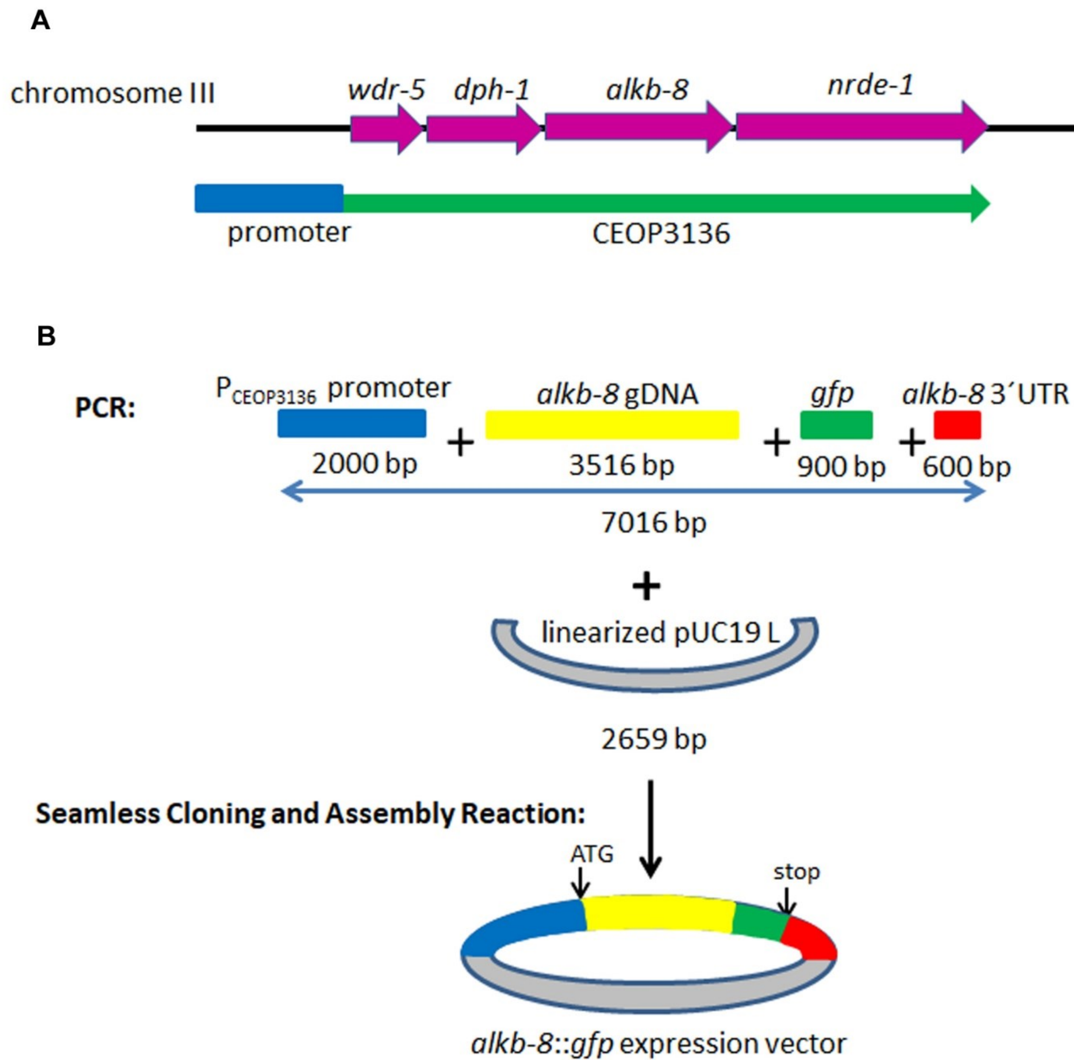

Fig. 2 Preparation of the transgenic line expressing *alkb-8::gfp* under the regulation of promoter of CEOP3136 and endogenous 3' UTR. A – Organization of *alkb-8* on chromosome III. *alkb-8* is the third gene in operon CEOP3136 and has its own internal promoter. B – Strategy for preparation of transgene expressing ALKB-8 tagged by GFP at its 3' end. Corresponding fragments of 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 Seamless Cloning Assembly Reaction.

Tab.1 List of used primers

primer name	5´→ 3´sequence	template		use
10/08	TCAAATTTTCTTCGCAATAATAATATAA	cDNA	AS	end of alkb-8 cDNA seq.
11/08	ATGTATTTCAATGAAGAAAAAGCGA	cDNA	S	start of alkb-8 cDNA seq.
12/08	CATTCAGCATTTGATGATCC	cDNA	S	real-time PCR primer
13/08	CCGTCTTTGAATTCCATTACTACA	cDNA	AS	real-time PCR primer
11/80	AATTCGAGCTCGGTACGGATAAGGAAGATCAT CAATGTTT	gDNA	S	CEOP3136 promoter
11/81	TCACACATATCTGAAATCACAGCAAAAATCAA	gDNA	AS	CEOP3136 promoter
11/82	TTTCAGATATGTGTGAGTTCATTTTTCAACCC	gDNA	S	alkb-8 gDNA
11/83	GGGTCCTCAATTTTCTTCGCAATAATAATATA	gDNA	AS	alkb-8 gDNA
11/84	AGAAAATTGAGGACCCTTGGAGGGTACCGGT A	plasmid DNA	S	gfp
11/85	TAAAAAAACTATTTGTATAGTTCATCCATGCC	plasmid DNA	AS	gfp
11/86	ACAAATAGTTTTTTTAAAGTTTTTTCTATTGG	gDNA	S	alkb-8 3'UTR
11/87	GCCAAGCTTGCATGCCTTTAGCGCAGTTTGAG AATCTGAA	gDNA	AS	alkb-8 3'UTR

2.5.5 Developmental assay

To estimate the timing of larval development of control *C. elegans* and animals with *alkb-8* downregulated by RNAi, the 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 lay eggs (after 78 hours at 16 °C) and the number of animals and laid eggs was determined on the photographs. The pictures were taken on Olympus SD30 microscope (Olympus, Tokyo, Japan) with Panasonic DMC-TZ3 camera (Panasonic, Kadoma, Japan).

2.5.6 Life span determination

For determination of life span, 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 *rol-6* gene as 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 life span in the overexpression experiment. In RNAi experiments 60 animals were followed in each group.

3 RESULTS

3.1 Whole genome expression analysis in animals without functional GEI-8 (CeNCOR)

3.1.1 Characterization of *gei-8* mutant strain

The *gei-8* mutant strain VC1213 was obtained from the *C. elegans* Gene Knockout Consortium and was backcrossed three times with adult wild type N2 males

and propagated as heterozygous animals carrying one normal *gei-8* allele and one *gei-8(ok1671)* allele with approximately one kilobase deletion. Amplification and sequencing of *gei-8(ok1671)* confirmed the 1095 bp deletion and 45 bp insertion that affects exons 7 and 8 of *gei-8a* isoform. RT-PCR and sequencing revealed a stop codon present in the *gei-8(ok1671*) transcript at position 663. The predicted protein contains SANT1 and SANT2 domains, but lacks the majority of the putative NR interaction sites at the C-terminus of the derived protein. The mutant mRNA was detected in homozygous *gei-8(ok1671)* animals using RT-qPCR at levels similar to wild-type animals, suggesting the truncated protein is likely to be expressed in both homozygous and heterozygous mutant animals. The overall normal heterozygous animals were indicating that the truncated GEI-8, if expressed at the corresponding protein level was not acting as a dominant negative regulator.

The homozygous *gei-8(ok1671)* animals were selected based on their characteristic phenotypes including a progressive locomotion defect, lower rate of pharyngeal pumping and a characteristic defect of gonad development. Homozygous mutants are sterile, with missing spermatheca, gonad arms are undeveloped, containing less meiotic nuclei and germ cells compared to control animals and distal tip cell (DTC) migration stopped early, reaching only two thirds of it's normal length of migration on the dorsal side of the animal. We concluded that the *gei-8(ok1671)* mutant germlines are arrested at L4 stage, before gonad elongation is completed and spermatheca is developed, although, some somatic markers of young adult stages are already present (adult vulva and adult alae). Mutant animals also have shorter maximum body length compared to wild type animals of the same age.

3.1.2 *gei-8* loss of function leads to transcription deregulation

Effects of the *gei-8(ok1671)* mutation on gene expression were studied with whole genome microarrays (Affymetrix). Changes in gene expression were defined as increased or decreased if statistically significant compared to wild-type controls in at least 2 out of 3 biological replicates (leaving the possibility of undetected change in one of three replicates provided that the third value was classified as undetected change but not as the opposite values of the remaining two replicates). Deregulated genes were analyzed for Gene Ontology (GO) term enrichment and clustered according to functional classification using DAVID 6.7 (Huang da et al.,2009a; Huang da et al.,2009b) and KEGG pathway tools (Arakawa et al.,2005).

Expression microarray analysis revealed 756 probe sets with decreased expression, corresponding with 690 unique Wormbase IDs. DAVID classification tools identified 645 IDs using medium classification stringency. GO analysis resulted in 32 clusters with an enrichment score greater than 2 and $P<0.05$. The list was enriched in spliceosome (29 genes), proteasome (13 genes), cysteine and methionine metabolism (7 genes), and RNA polymerase genes (6 genes) as identified by KEGG pathway analysis. Among specific genes involved are RNA polymerase II and III (Pol II subunits B4, B7, B9 and Pol III subunits AC2 and F09F7.3), spliceosome components (U1 to U6 snRNAs, *hel-1* helicase and others), and proteasome subunits (*pas-3*, *pas-4*, *pbs-1*, *pbs-3*, *pbs-4*, *pbs-6*, *pbs-7*, *rpt-1, rpt-2*, *rpn-2*, *rpn-5*, *rpn-8*, *rpn-12*). The most common functional categories over-represented by the changes in gene expression were growth, embryonic or larval development and development of reproductive structures. Other clusters include multiple histones and histone-like genes, mitochondrial membrane proteins, sperm structural proteins and hedgehog-like family genes. Interestingly, the set of genes downregulated in *gei-8* mutants included several genes required for proper

muscle function, including *unc-52* (myofilament assembly and/or attachment of the myofilament lattice to the cell membrane), *unc-27* (troponin I family), *unc-54* (muscle myosin class II heavy chain), *pat-10* (body wall muscle troponin C), *lev-11* (tropomyosin), *mlc-2* (myosin light-chain), and *tni-1* (troponin 1). It is unclear if such changes in muscle gene expression contribute to, or are the result of, the defective movement phenotypes we observed in *gei-8(ok1671)* mutant animals. Depletion of NCOR1 function specifically in mouse muscle resulted in increased muscle mass and mitochondrial function (Yamamoto et al.,2011), a phenotype opposite to what we observed in worms with reduced GEI-8 activity in all tissues.

Microarray analysis revealed 296 probe sets with increased expression, corresponding to 275 unique Wormbase IDs. GO analysis identified 7 clusters with an enrichment score greater than 2 and $P<0.05$. Enriched clusters included gene annotations for life span and aging, lipid transport and vitellogenin genes, stress response (heat shock and cellular stress), metabolic genes (sugar metabolism, glycolysis), and neuropeptide signaling (including genes coding for neuropeptide like proteins *nlp-27* to *nlp-32*). The KEGG pathway analysis identified six groups including genes involved in glycolysis (8 genes), cystein methionine metabolism (4 genes), galactose metabolism (3 genes), pentose phosphate pathway (3 genes), fructose and mannose (3 genes) and tryptophan metabolism (3 genes).

3.1.3 21U-RNAs are involved in the defective development of gonad

One of the most significantly affected genes in the *gei-8(ok1671)* homozygous mutants was Y9C9A.16, encoding a predicted mitochondrial sulfide:quinone oxidoreductase, which had an averaged 7.6-fold increase in expression compared to wild-type controls. This increase was confirmed by RT-qPCR. The Y9C9A.16 region is

assayed by Affymetrix probe set 184710_at and, interestingly, includes three 21U-RNAs: 21ur-2020, 21ur-11733 and 21ur-9201. To determine if disruption of expression of Y9C9A.16 affect development, we performed RNAi targeted to the spliced mRNA covered by the Affymetrix probe set (184710_at) or only the regions that include 21ur-2020, 21ur-11733 and 21ur-9201. Progeny of parental animals injected with dsRNA targeting the specific regions were scored using Nomarski optics and fluorescent microscopy (DAPI stained). We were not able to identify any specific phenotype of Y9C9A.16 knockdown in wild type animals. However, because the expression from Y9C9A.16 showed a dramatic response to loss of GEI-8 activity, we thought there might be a biological connection between them. We predicted that knockdown of the expression from Y9C9A.16 locus in *gei-8 (ok1671)* homozygous mutants might revert or modify some of the observed phenotypes; the latter was observed. RNAi-mediated knockdowns targeted to the region covered by the 184710_at probe set and the region containing 21ur-2020, 21ur-11733 and 21ur-9201 induced additional phenotypes in the *gei-8(ok1671)* homozygous mutant background. Additional phenotypes included severe distal tip cell migration defects, irregular gonadal nuclei tumor like accumulation of germline cells and vulval protrusions were observed in 13.9% of homozygous *gei-8(ok1671)* animals treated with Y9C9A.16 RNAi (n=481). Interestingly, Y9C9A.16 has a paralogue in the *C. elegans* genome, the gene *sqrd-1* (**s**ulfide:**q**uinone oxido**red**uctase). This gene encodes a protein that is identical in size (361 aa) to Y9C9A.16 sharing 266 identical amino acids in its sequence although the genes share very little DNA homology includes numerous 21U-RNAs. RNAi targeted to unique regions of the *sqrd-1* coding region, including four 21U-RNAs, resulted in changes in gonad arm migrations and an accumulation of germline cells (4.5% affected, n=198) that were similar, although less severe, as those observed after Y9C9A.16 RNAi. We

concluded that the paralogues encoded by Y9C9A.16 and *sqrd-1*, and perhaps their associated 21U-RNAs, have overlapping roles during development of the germline that can be exacerbated by loss of GEI-8 activity.

3.2 Characterization of ALKB-8 in *C. elegans*

3.2.1 Analysis of the expression of *alkb-8* by RT-qPCR

We analyzed the gross expression of *alkb-8* during developmental stages using reverse transcription-quantitative PCR. The results were normalized for the expression of Polymerase II subunit *ama-1* and related to the expression observed in mixed stages embryos. The relatively high expression of *alkb-8* in embryos decreased in synchronized L1 larvae and steadily increased from L2 stage to young adults (Fig. 3a). We also analyzed the effect of 6 hours fasting and feeding in synchronized L1 larvae on 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 while *fat-7* and *asc-2* expression responded to fasting as expected.

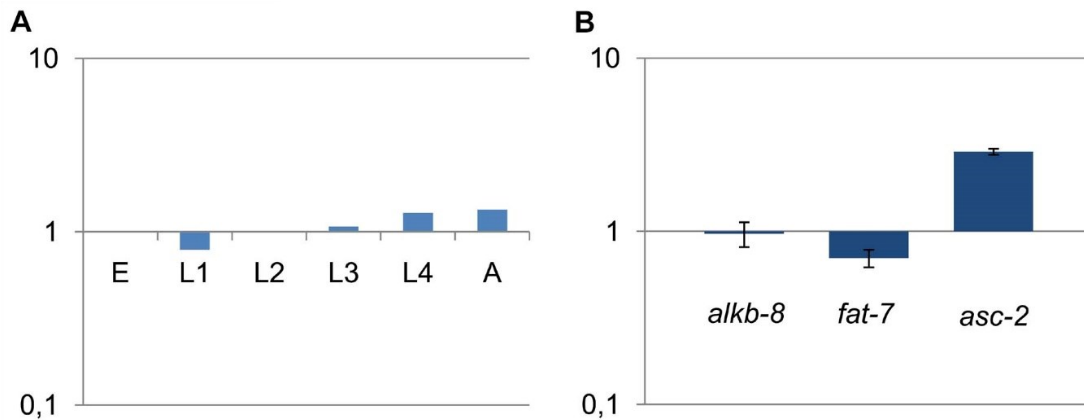

Fig. 3 The expression profile of *alkb-8* gene analyzed by RT-qPCR. A-The expression of *alkb-8* during development. Results are shown in logarithmic scale and the values represent fold change of expression compared to expression in embryos. The expression drops in the L1 stage and gradually increases during development. B- The relative expression of *alkb-8* after six hours of fasting. The values represent fold change of expression compared to 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* (expression decreases after fasting) and *asc-2* (increases) were used for control.

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

According to WormBase (WS263) *alkb-8* is organized as a third gene in a hybrid operon CEOP3136 indicating that its expression depends partially on the operon promoter and partially on its own promoter. Expression of *alkb-8* dependent on the internal promoter was described by Pastore and coworkers 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 (Pastore et al.,2012). For visualization of *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 time exposure 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 (Fig. 4 and Fig. 5). We also observed diffuse expression in intestinal cells (Fig. 4).

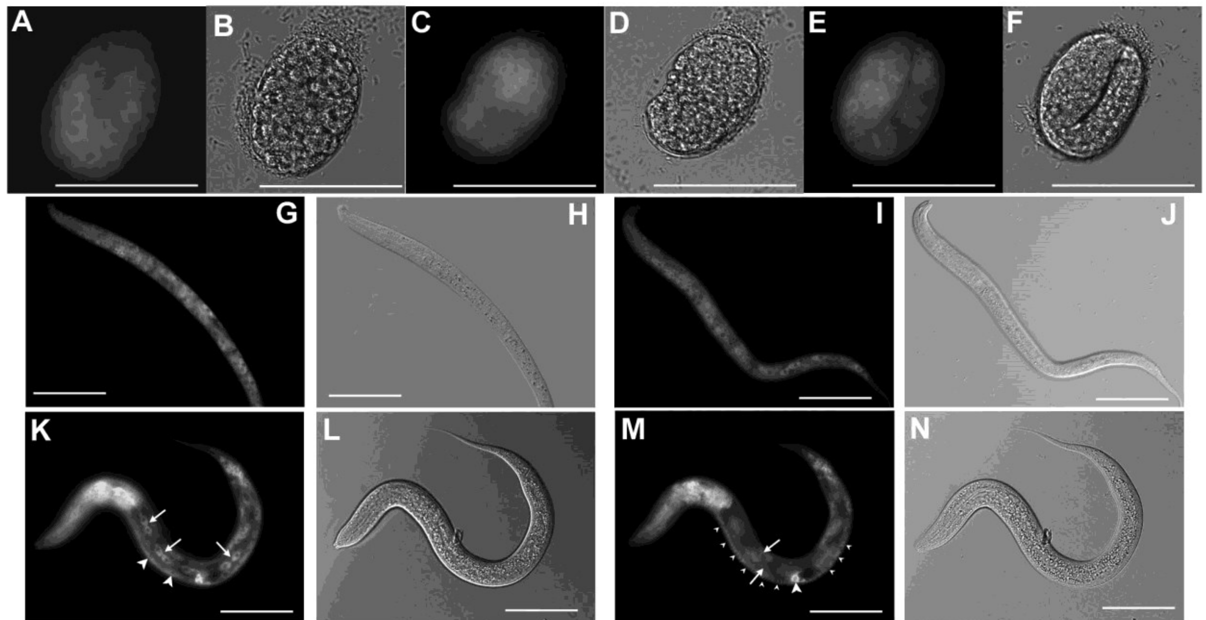

Fig. 4 Expression pattern of ALKB-8::GFP in early stages of development using a transgenic line carrying an extrachromosomal DNA construct.
The construct composition is shown in Fig. 2. The GFP signal in embryos can be detected early after eggs are laid (around 40 cells stage) shown in panels A and B. 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 as shown 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. Bar represent 50 µm.

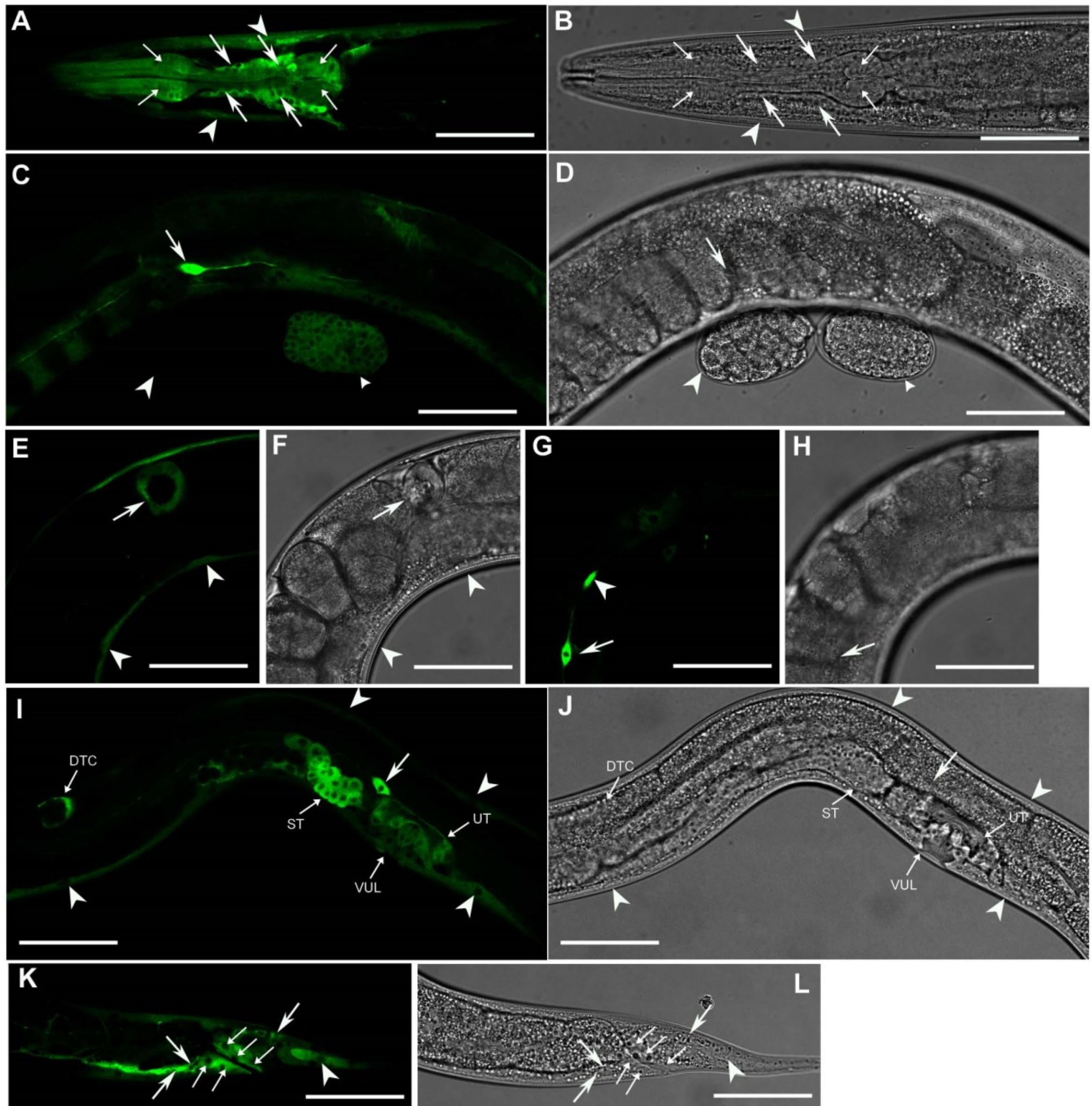

Fig. 5 Expression of *alkb-8::gfp* from extrachromosomal arrays regulated by the promoter of CEOP3136 operon analyzed by confocal microscopy. Panel A shows the expression of *alkb-8::gfp* in the head of an adult animal. Strong signal is detected in neurons (arrows), pharyngeal muscle cells (small arrows) 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 in the left is approximately in the 30 cells stage (arrowhead) and shows no expression of *alkb-8::gfp*. In contrast, the embryo on the right is in the approximately 100 cells stage (small arrowhead) and shows ubiquitous cytoplasmic expression of the transgene. The canal-associated neuron (CAN) marked by 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 a 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 a 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 shows the same picture as the fluorescent picture on their left in Nomarski optics. Bars represent 50 µm.

3.2.3 The effects of *alkb-8* downregulation 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 contrary, 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 was not causing delays in complete larval stages, we analyzed the onset of egg laying in control and RNAi treated cultures. This strategy revealed clearly observable difference in time given by the onset of egg laying by control larvae at which the larvae with downregulated *alkb-8* laid already approximately 50 times more embryos (Fig. 6). No specific developmental defects were observed.

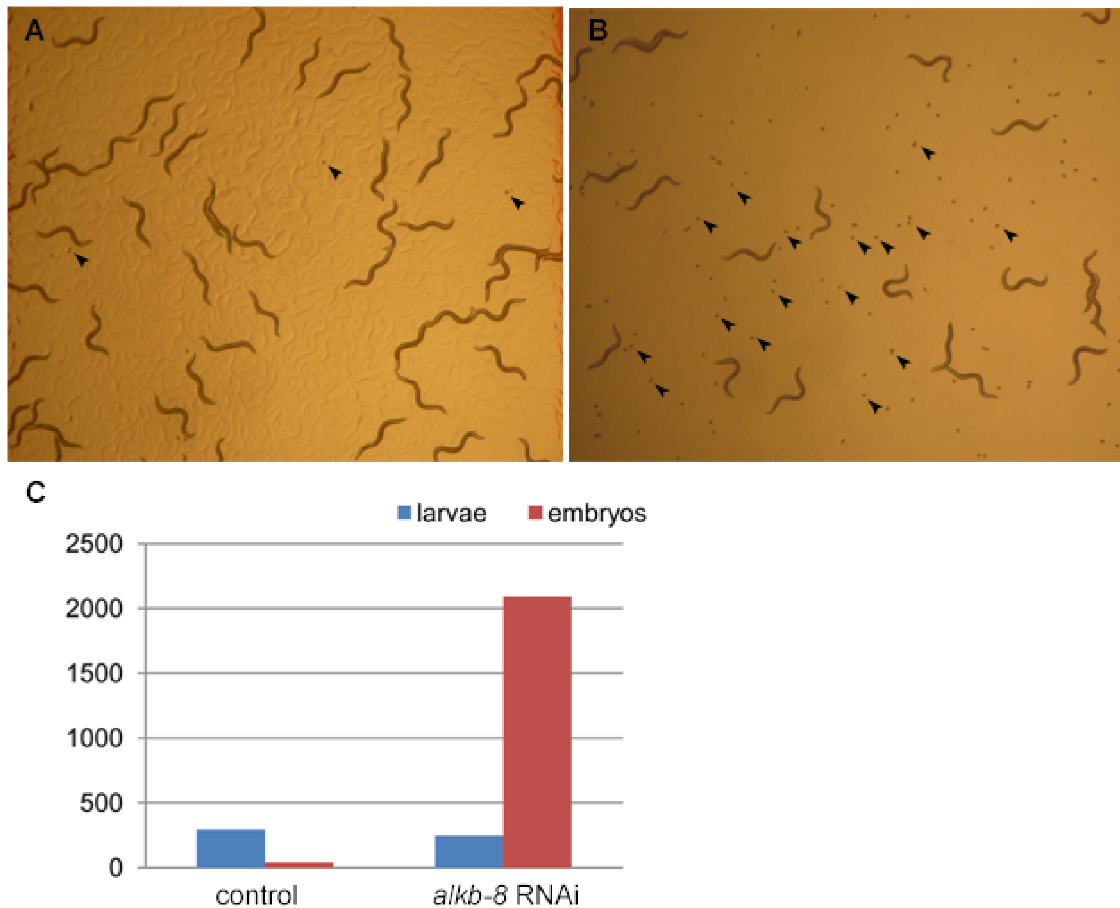

Fig. 6 Analysis of the effect of alkb-8 downregulation by RNAi on *C. elegans* larval development. Equal amounts of synchronized L1 larvae were transferred on plates with control cultures (HT115 bacteria transformed with empty L4440 vector) and experimental plates seeded with bacteria transformed with 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 start to lay eggs. At this time, equal areas of plates with nematodes were photographed and the number of animals (and laid eggs) was determined. Panel **A**- shows section of control plate, **B**- shows section of plate seeded with bacteria expressing *alkb-8* dsRNA. Arrowheads poit to laid embryos (not all embryos are indicated). In panel **C**- total number of larvae and embryos counted in one plate from both experimental conditions is showed. 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 contrary, animals with downregulated *alkb-8* developed faster compared to control animals**.**

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

In order to assess a 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 analyzed 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. 7).

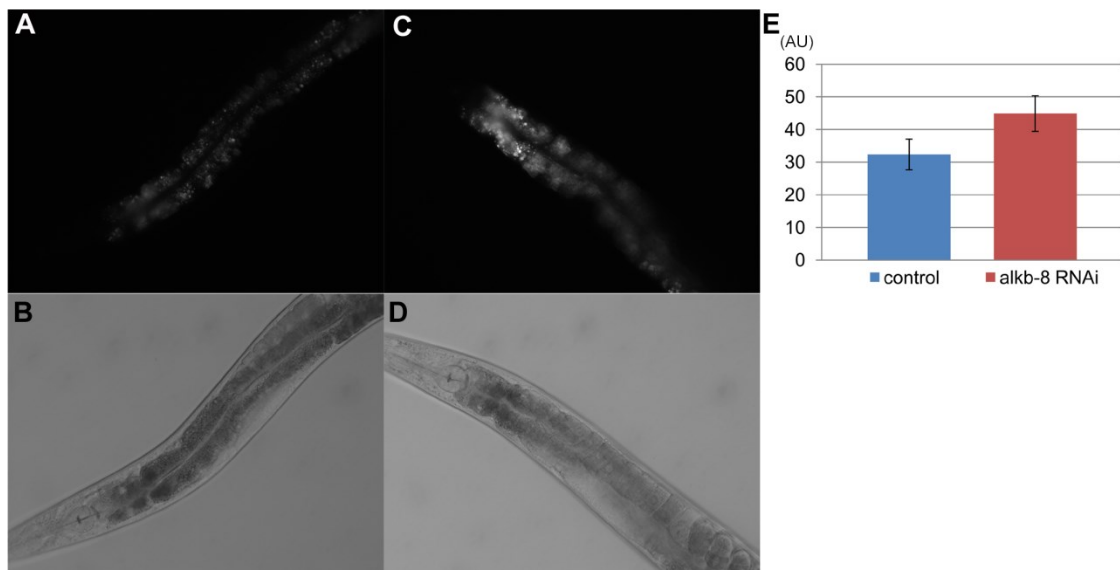

Fig. 7 Detection of the signal in the *in vivo* Nile red stained compartment in control animals and animals with downregulated *alkb-8*. Panel **A** shows a Nile red derived fluorescence in a young adult control animal. Panel **B** shows the same animal in Nomarski optics. **C**- shows a larva with *alkb-8* inhibited by RNAi with the identical optical settings (same as C in Nomarski optics). Panel **E** 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* down regulated by RNAi compared to control animals. $P < 0{,}001$.

We also assayed if 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 plasmid pPD49.78 and 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 case of pPD49.83. Both transgenic lines showed a strong decrease in the extent of Nile red positive signal in enterocytes (Fig. 8). Keeping with ALKB-8 intestinal role, the line based on pPD49.83 which leads to a strong intestinal expression of the transgene showed the lowest values for Nile red dependent fluorescence.

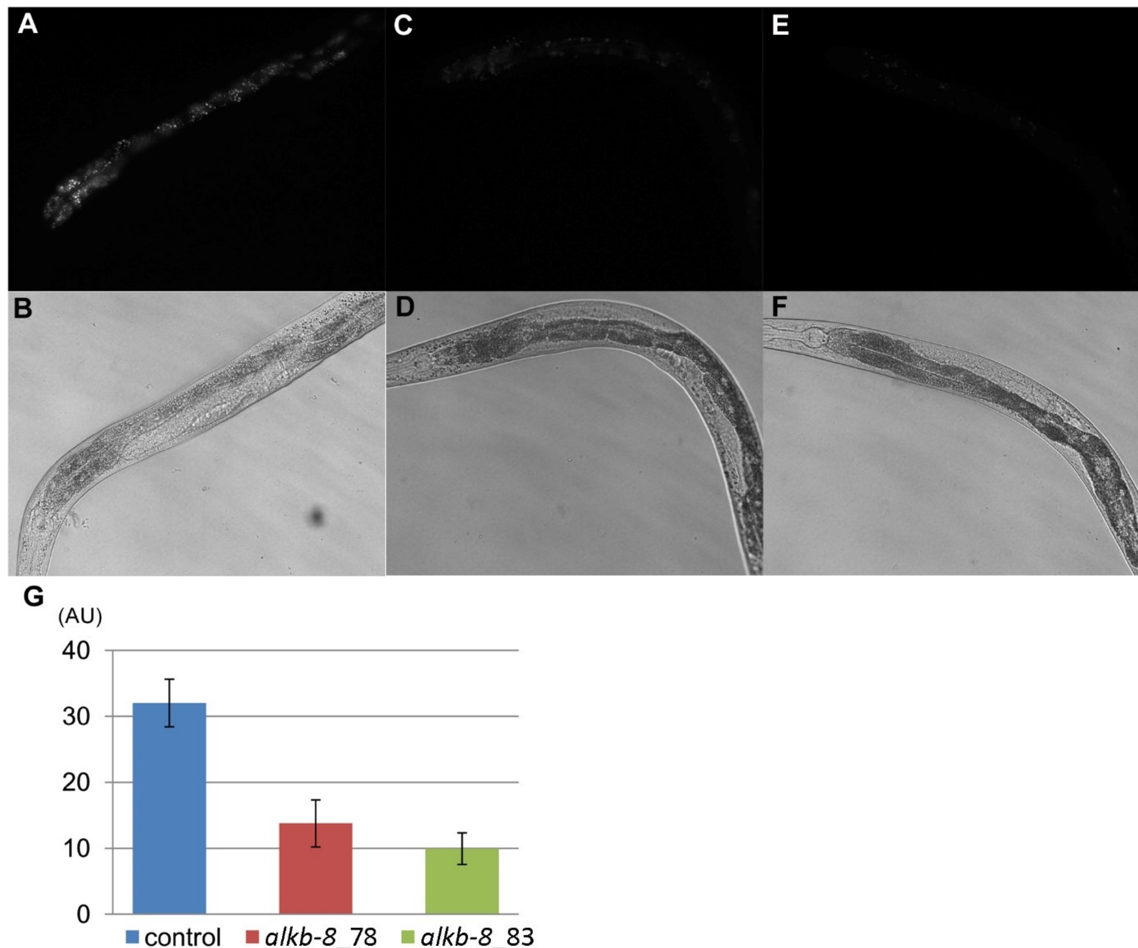

Fig. 8 The effect of *alkb-8* forced overexpression on the signal of the Nile red positive compartment of LRO. Panels A, C and E show fluorescence images of young adult larvae stained in vivo with Nile red. Panel A- shows an animal from the control group, panel C- an animal from the group overexpressing *alkb-8* from pPD49.78 vector, panel E- an animal from the group with *alkb-8* in pPD49.83 vector. Panels B, D and F show the same pictures as the pictures next to them 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 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

3.2.5 The effect of *alkb-8* overexpression on *C. elegans* life span

To determine if the effect of ALKB-8 on the Nile red positive compartment has a broader metabolic role, we assayed the life span of animals with downregulated alkb-8 expression or pulse-overexpressed alkb-8. Downregulation of alkb-8 expression

(applied for the entire lifetime of the assayed animals) had no effect on the animal life span (Fig. 9). In strong contrast, pulse forced expression in animals during their L1 stage led to pronounced life span extension of experimental animals reaching 10 to 40%.

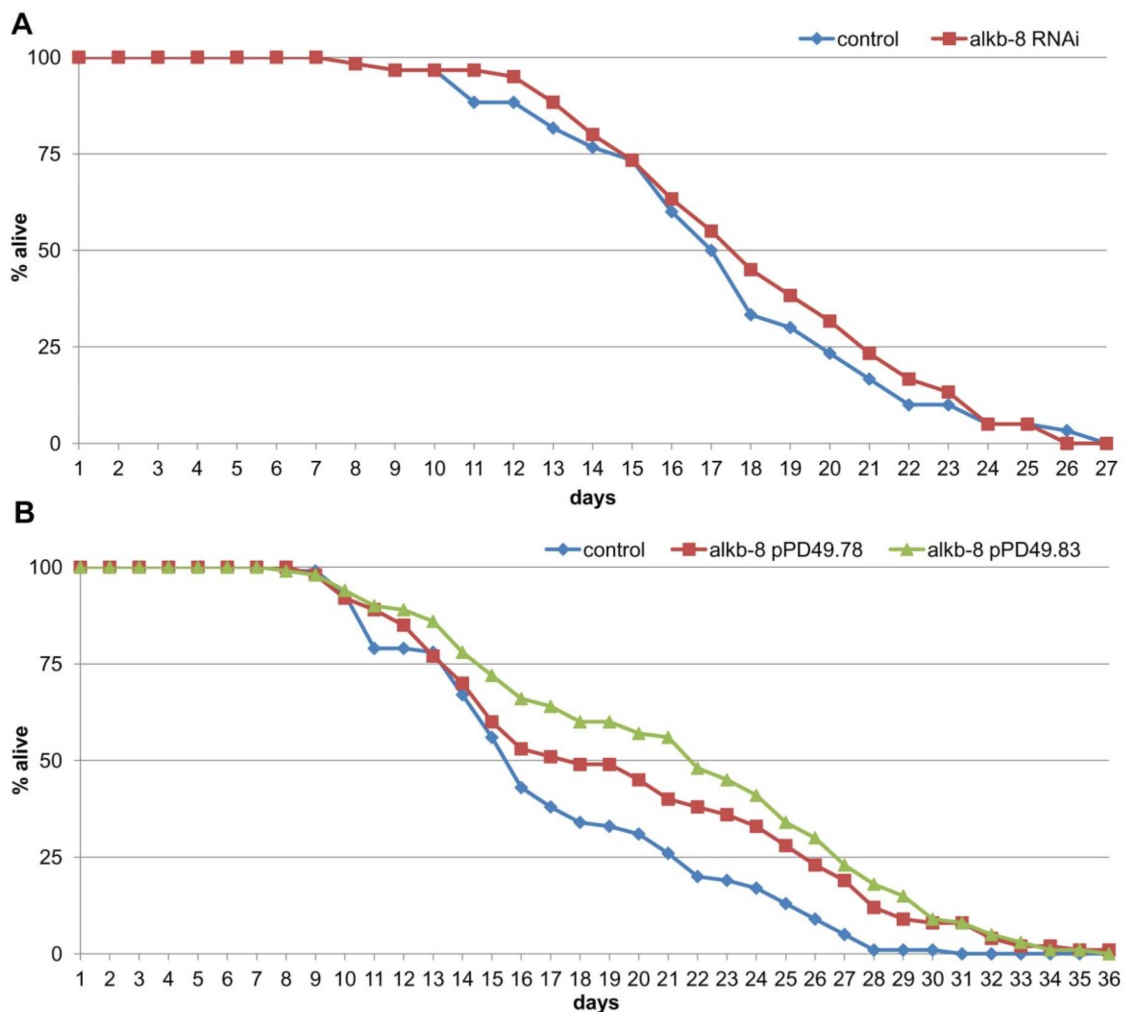

Fig. 9 Determination of the effect of *alkb-8* on the life span of *C. elegans*. Panel A – The effect of *alkb-8* downregulation on nematode longevity. Animals inhibited for *alkb-8* to the level that is affecting Nile red positive compartment staining has no effect on nematode longevity. Panel B – the effect of pulse overexpression in L1 stage on *C. elegans* longevity. Compared to controls, animals with forced expression of *alkb-8* have life span extended by 10 to 40 %.

4 DISCUSSION

Our reports connected with this thesis showed that posttranscriptional modifications modulate very importantly gene expression. We have used two model situations for their visualization.

4.1 GEI-8 loss of function leads to transcriptional deregulation

Firstly, we analysed a differential display of whole genome expression in homozygous animals carrying a large deletion in *gei-8* gene (allele *gei-8(ok1671)*). This allele is likely to be functionally null mutant. Technically, some mRNA allowing the production of a C-terminally abrogated protein may have been transcribed. It is, however, likely that such protein if produced at all, would be formed in low numbers because of premature stop codons present in mRNA are likely to result in mRNA degradation based on nonsense mediated decay. The resulting protein would lack all sequences necessary for the interaction with transcription factors. The remaining protein fragment may activate HDACs (especially HDAC3) but it would be unable to localize it to functionally relevant chromatin domains.

The analysis uncovered a relatively small set of genes with increased expression that did not show functional connections and much larger set of genes with decreased expression, that were functionally clustered, and were corresponding to the observed phenotype. The phenotype that is critical for the topic of this thesis is the defective gonad development and a complete sterility of homozygous animals.

This seems to be very difficult to explain for a protein that is proven transcriptional repressor with no known gene activation functions. There is, however, one report that documents NCORs' presence in genomic localizations that are

transcriptionally active in response to 1,25(OH)2D3 and associate with transcription activators SRC1, CBP and MED1 (Meyer and Pike,2013).

Silenced or inhibited gene expression in response to the absence of a GEI-8 repressor be explained by the following possibilities:

1. GEI-8 may repress another repressor. Its de-repression may lead to decreased expression of downstream genes.
2. GEI-8 may be also a transcriptional activator. Its elimination would lead to decreased expression of transcriptionally dependent genes.

The situation is to some extent clarified by the results of microarrays that visualize all genes (present on Affymetrix microarrays) and are likely to uncover both strong transcriptional repressors (as well as activators). Nevertheless, changes of expression of genes coding for strong repressors and activators were not identified in the *gei-8* mutant animals.

4.2 21U-RNAs of piRNA class are likely to be responsible for *gei-8(VC1213)* gonadal phenotype

Since we did not identify genes coding for protein that would explain the repressive effect of GEI-8 loss of function, we turned our attention to possible regulatory RNAs. One gene that was identified as strongly transcriptionally increased in *gei-8(ok1671)* mutants - Y9C9A.16 encodes a sulfide:quinone oxidoreductase that we named *sqrd-2* based on its homology to known sulfide:quinone oxidoreductase *sqrd-1*. Keeping with the pathogenic function of *sqrd-2* derepression in *gei-8* mutant animals, we showed that inhibition of *sqrd-2* in homozygous mutants *gei-8(ok1671)* induces partial reversal of *gei-8* mutant phenotype. We also demonstrated similar reduction-of-

function phenotypes for the *sqrd-2* paralogue, *sqrd-1*. Both *sqrd* genes include 21U-RNAs scattered throughout their non-coding regions. 21U-RNAs have been shown to be critical for sperm development and transposon silencing (Batista et al.,2008). Both *sqrd* genes may be linked to their associated non-coding 21U-RNAs that may be localized in mitochondria as part of piRNA biosynthesis (Huang et al.,2011). Changes in the mitochondrial compartment induced by *gei-8* inhibition as reported by Yamamoto et al. (Yamamoto et al.,2011) and observed in our experiments on *gei-8(ok1671)* mutants may also involve piRNAs mediated regulation. The role of 21U-RNAs in *gei-8(ok1671)* is supported by our findings that additional changes in the phenotypes of homozygous mutants *gei-8(ok1671)* are induced by RNAi targeted at *sqrd-1* gene. One of the three isoforms of *sqrd-1* is predicted to code for a protein with the same length as the protein derived from *sqrd-2* and both proteins show 74% identity in amino acid sequences. It seems likely that *sqrd-2* and *sqrd-1* can substitute for each other in function. 21U-RNAs located in *sqrd-2* show approximately 50% identity in the conserved cores formed by 16 or 17 bases with piRNAs found in *sqrd-1*. We hypothesized that the levels of the non-coding RNAs located within *sqrd-1* and *sqrd -2*, as well as the function of SQRD-1 and SQRD-2 may be critical for gonad development. At present, it is impossible to determine if mammalian NCORs are also regulating gonad development similarly as GEI-8 since both NCOR1 and NCOR2 are embryonically lethal (Jepsen et al.,2000; Jepsen et al.,2007). Nevertheless, 21U-RNAs are regulated by fork-head transcription factors (Cecere et al.,2012) and the fork-head factor FoxP1 regulates development in concert with SMRT (Jepsen et al.,2008). Critical role for piRNA in regulation of cell differentiation is emerging from studies indicating that increased expression of specific piRNAs is linked to cancer development (Lim et al.,2014; Weng et al.,2018) and organogenesis (Vella et al.,2016).

4.3 ALKB-8 2-oxoglutarate and Fe^{2+} dependent dioxygenase and TRM-9 related methyltransferase regulates biology of Nile-red in vivo stained lysosome-related organelles (LRO) in *C. elegans*

Our results support ALKB-8 modulatory function in metabolic events linked to lysosome-related organelles in *C. elegans*. Surprisingly, despite that *alkb-8* being expressed strongly and ubiquitously from early embryonic stages to adulthood, its downregulation by RNAi to levels that affect the 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. Keeping with the metabolic roles of ALKB-8, its overexpression applied during the first larval stage markedly prolonged life span. On the other hand, downregulation of *alkb-8* by RNAi does not shorten their life span. There are several factors that may cause this discrepancy. Firstly, RNAi is not significantly affecting neuronal cells in wild type N2 *C. elegans* 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.

4.4 ALKB-8 regulates lifespan in *C. elegans*

The effect on longevity may be to a large extent based on neuronal functions of ALKB-8. In agreement with this, in *rrf-3* mutant animals, in which RNAi affects also

neuronal cells, neuronal inhibition of the autophagy nucleation complex extends life span 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 *C. elegans* life span and that post-reproductive inhibition of *bec-1* mediates longevity specifically through the neurons (Wilhelm et al.,2017).

The positive effect of ALKB-8 on life span 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 minutes in the L1 larval stage and the life span of control animals subjected to the short-term heat-shock did not differ from the normal life span of animals kept under similar laboratory conditions but not subjected to the experimental heat-shock. 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 (Cunniff et al.,2014; Li et al.,2012).

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 in chromosome III in a hybrid operon CEOP3136. As such, it is trans-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 (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 a yeast methyl transferase TRM9 (TRM9_YEAST) not only in the SAM binding part but also at the C-terminus. Deletion of TRM9 significantly increased life span 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 to protect 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*, that only has the methyl transferase domain, not the demethylase domain C35D10.12 (NP_497751.1) but nothing is known about its function.

5 CONCLUSIONS AND EVALUATION OF GOALS AND HYPOTHESES

We hypothesized that the model of C. elegans may be used for visualization of posttranscriptional mechanisms that regulate gene expression in response to developmental and metabolic needs. To achieve this, we set our goals to analyze the consequences of loss of function of a major transcriptional repressor, GEI-8/NCOR1 and search for functional connection between whole genome expression and a phenotype of GEI-8 loss of function, which includes defective gonad development and sterility. Our results suggest that the gonadal mutant phenotype is caused by elevated expression of 21U-RNAs of piRNA class. This adds important data for understanding biogenesis of 21U-RNAs and their involvement in the gonad development.

The second goal was to functionally analyze ALKB-8, the nematode orthologue of ALKBH8. The work on this project led to identification of ALKB-8 role in regulation of lysosome related organelles and lifespan in *C. elegans* what attributes new functions for ALKBH8 orthologues.

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Supplementary files

1) Kollárová J, Kostrouchová M, Benda A, Kostrouchová M. (2018) 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. *Folia Biologica* (in press)

2) Mikoláš P, Kollárová J, Sebková K, Saudek V, Yilma P, Kostrouchová M, Krause MW, Kostrouch Z, Kostrouchová M. (2013) GEI-8, a Homologue of Vertebrate Nuclear Receptor Corepressor NCoR/SMRT, Regulates Gonad Development and Neuronal Functions in Caenorhabditis elegans. *PLoS One*. 8(3):e58462.