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Regulation of gene expression at posttranscriptional levels

Regulace genové exprese na posttranskripčních úrovních

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CONTENT

ABSTRACT 4
ABSTRAKT 4
1 INTRODUCTION 5
1.1 Regulation of gene expression 5
1.1.1 Regulation of gene transcription 5
1.1.2 Regulation on posttranscriptional levels 7
1.2 The model organism Caenorhabditis elegans as powerful tool for studies of gene expression regulation 10
2 HYPOTHESES 11
3 GOALS 12
4 MATERIALS AND METHODS 12
5 RESULTS 12
5.1 Whole genome expression analysis in animals without functional GEI-8 (CeNCOR) indicated 21U-RNAs of piRNA class to be responsible for the phenotype of defective gonad development 12
5.2 ALKB-8, the nematode orthologue of ALKBH8 regulates Lysosome-related organelles and lifespan in C. elegans 14
5.2.1 ALKB-8 regulates lysosome related organelles visualized by in vivo Nile red-staining 14
5.2.2 ALKB-8 regulates life span in C. elegans 15
6 DISCUSSION 16
6.1 GEI-8 loss of function leads to transcriptional deregulation on the whole genome scale 16
6.2 21U-RNAs of piRNA class are likely to be responsible for gei-8(VC1213) gonadal phenotype 17
6.3 ALKB-8 2-oxoglutarate and Fe2+ dependent dioxygenase and TRM-9 related methyltransferase regulates biology of Nile-red in vivo stained lysosome-related organelles (LRO) in C. elegans 17
6.4 ALKB-8 regulates lifespan in C. elegans 18
7 CONCLUSIONS AND EVALUATION OF GOALS AND HYPOTHESES 19
References 20

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 pravděpodobným 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.

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 [1].

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 [2, 3] and regulatory RNAs including miRNA[4], siRNA[5], piRNA [6] and lncRNA RNA classes [7] 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 [8, 9].

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 [10]. Approximately 2000 genes are recognized as transcription factors in human genome [11]. 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 [12], and SP1 and nuclear receptors in mammalian cells (reviewed in [13]). 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 [14]). 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 [15, 16].

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 [17-27]. 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 [17, 21, 24, 28, 29]. The CDK module is dispensable for gene expression activation and has in most cases repressive role in Mediator complex mediated transcription [21].

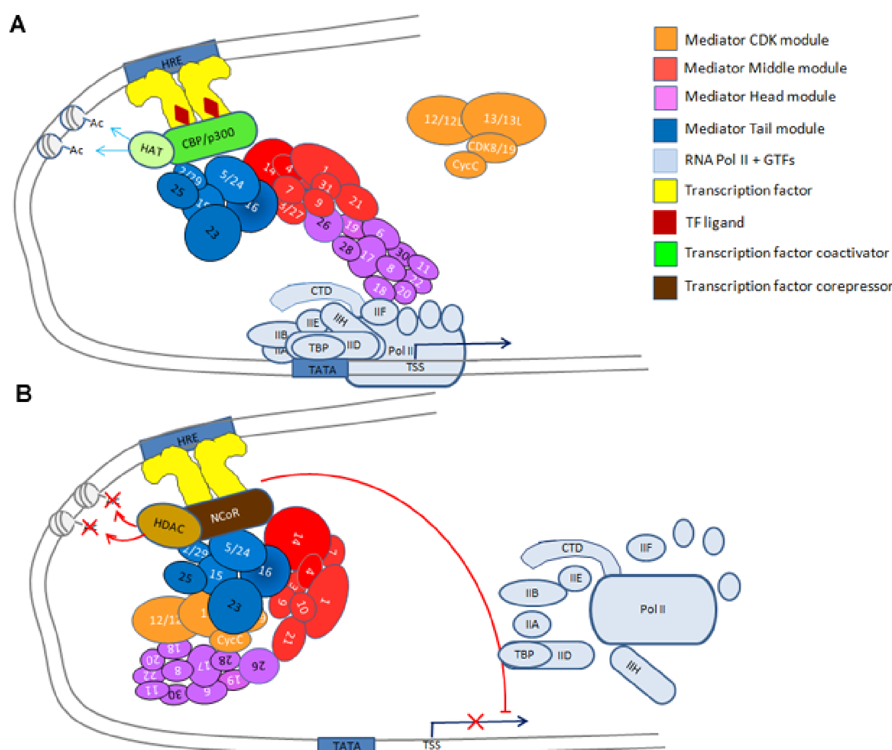

Fig. 1 Simplified scheme of regulation of gene transcription by nuclear receptors. Panel A – Schematic representation of transcription activation by a liganded nuclear receptor(s). Association of the activating ligand results in conformational change of the ligand binding domain of nuclear receptors 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 [21, 30].

NCoR and SMRT are paralogous vertebrate proteins that were first identified as transcriptional corepressors interacting with unliganded nuclear receptors (thyroid and retinoid receptors) [15, 16]. 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.
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..
The NCOR nematode orthologue GEI-8 was originally identified as a GEX-3 binding protein based on yeast-two-hybrid assays [31]. 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 [32, 33]. GEI-8 thus offers new possibilities for functional analyses of NCOR role in biology of nematode species.

1.1.1.1 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) [34, 35].

1.1.1.2 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. 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 [36, 37]. 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 [38, 39].

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 [40]. 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. Posttranscriptional regulation of gene expression includes capping of 5' end of mRNA, splicing of mRNA and its 3' end polyadenylation, RNA editing (cytidine conversion to uridine (C-to-U), dependent APOBEC ("apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like") and deamination of adenosine leading to formation of inosine which dependent on the ADAR ("adenosine deaminase acting on RNA") enzyme gene family [41, 42].

Posttranscriptional chemical modification of ribonucleotides is another way how to affect stability and function of RNA molecules. 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 [43].
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 [44, 45]). First were discovered demethylases of m^6A: FTO [46] and ALKBH5 [47], 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 [48]. 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 [44]. 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 [45].
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 [45, 49].
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 [50].
The most heavily modified RNA species is **tRNA**, where over 80 different modifications were found [51]. 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 [52].
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 [45, 53, 54]. Function of wobble uridine modification

introduced by enzyme ALKBH8 (ALKB-8 in *C. elegans*) is described in more detail in chapter ALKB-8 introduction

1.1.2.1 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 [55].
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.
Regulatory RNAs include 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) [56, 57]. 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 [58].
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 [59]. 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 [55]).
siRNA as a separate class of RNAi were 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 [60]. 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 [55].
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 [55].
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 [32], 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 eukaryotic RNAs were recently described (reviewed in [55].

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 obtained experimetnal data with a fast growing knowledge gained by the whole scientific community and shared through the bioinformatic tool named WormBase.

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.

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 [61-63].

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 [62, 64, 65]. *FTO* gene received attention for its association with human obesity [66, 67] later in part shown to be associated with a homeobox gene IRX3 that is regulated by noncoding sequences within the *FTO* gene [68]. 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 [69].

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 [70, 71]. 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 [63, 71]. 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 [72]. 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 [73, 74]. The AlkB domain of ALKBH8 specifically hydroxylates mcm(5)U into (S)-mchm(5)U diastereomer in tRNA-Gly(UCC) [63].
The ALKBH8 methyltransferase domain shows close relationship to a yeast methyltransferase TRM9. The function of the yeast TRM9 has been investigated in [75, 76]. 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 [77-79]. This mechanism is conserved from protozoa, to animals and plants.

ALKBH8 was shown to regulate selenocysteine-protein expression as a protective mechanism against damage by reactive oxygen species [80]. *C. elegans* has two thioredoxin reductases, TRXR-1 and TRXR-2 [81] 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* [82, 83]. 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 [84].

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 [85]. *In vivo* Nile red uptake may be used as an effective tool for identification of proteins that function at the level of specific LRO [85].

2 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.

3 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.

4 MATERIALS AND METHODS

Projects linked to this thesis were done on the *C. elegans* model organism and employed its powerful genetic and genomic methods. The employed methods included:

Generation of transgenic strains of C. elegans, cultures of mutant animals obtained from the *C. elegans* Gene Knockout Consortium, advanced microscopy and microinjections. The work further included general molecular biology methods (purification of nucleic acids, reverse transcription of RNA into cDNA, amplification by polymerase-chain reaction in the end-point as well as quantitative setting, DNA sequencing, RNA interference, whole genome expression analysis using microarrays and advanced bioinformatics. Methods are described in detail in publications associated with this thesis and are accessible online (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0058462; methods concerning ALKB-8 will be accessible at https://fb.cuni.cz/2018).

The microarray data has been deposited in the NCBI's GEO database (http://www.ncbi.nlm.nih.gov/geo) accession number GSE40127.

5 RESULTS

5.1 Whole genome expression analysis in animals without functional GEI-8 (CeNCOR) indicated 21U-RNAs of piRNA class to be responsible for the phenotype of defective gonad development

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.

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.

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 [33], 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).

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.

5.2 ALKB-8, the nematode orthologue of ALKBH8 regulates Lysosome-related organelles and lifespan in C. elegans

First, we established the expressional pattern of *alkb-8* during developmental stages using reverse transcription-quantitative PCR and transgenic animals 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.

5.2.1 ALKB-8 regulates lysosome related organelles visualized by in vivo Nile red-staining

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. 2).

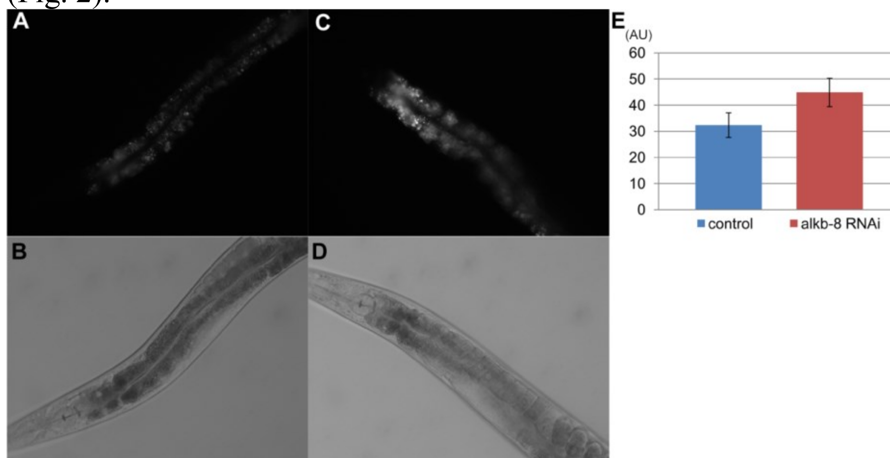

Fig. 2 Detection of the signal in the in vivo Nile red stained compartment in control animals and animals with downregulated alkb-8. Panel A shows a Nile red derived fluorescence in a young adult control animal. Panel B shows the same animal in Nomarski optics. C- shows a larva with alkb-8 inhibited 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. 3). 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.

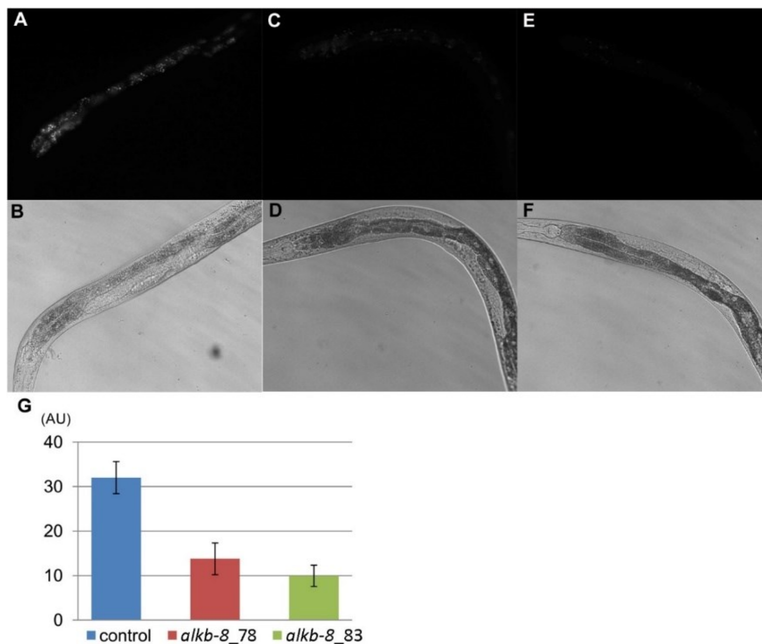

Fig. 3 The effect of alkb-8 forced overexpression on the signal of the Nile red positive compartment of LRO. Panels A, C and E show fluorescence images of young adult larvae stained in vivo with Nile red. Panel A- shows an animal from the control group, panel C- an animal from the group overexpressing 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. $P < 0,0001$

5.2.2 ALKB-8 regulates life span in C. elegans

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. 4). 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%.

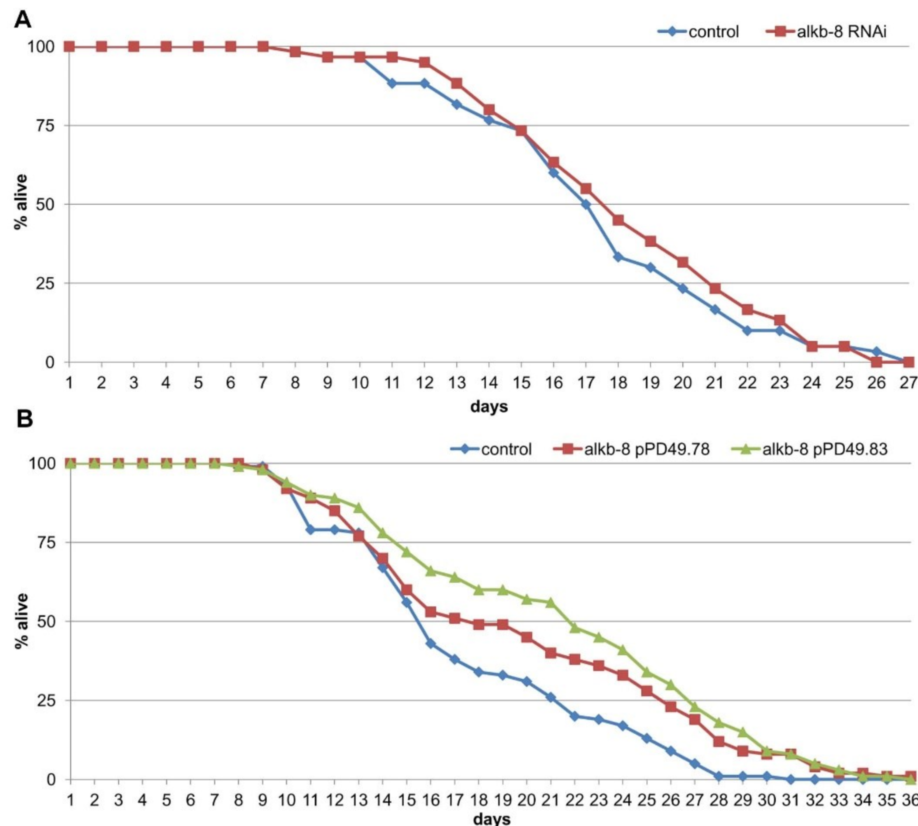

Fig. 4 Determination of the effect of alkb-8 on the life span of C. elegans. Panel A – The effect of alkb-8 downregulation on nematode longevity. Animals inhibited for alkb-8 to the level that is affecting Nile red positive compartment staining has no effect on nematode longevity. Panel B – the effect 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 %.

6 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.

6.1 GEI-8 loss of function leads to transcriptional deregulation on the whole genome scale

The observed situation, which 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 a 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 [86].

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, genes coding for strong repressors and activators were not identified in the *gei-8* mutant animals.

6.2 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 [87]. Both *sqrd* genes may be linked to their associated non-coding 21U-RNAs that may be localized in mitochondria as part of piRNA biosynthesis [88]. Changes in the mitochondrial compartment induced by *gei-8* inhibition as reported by Yamamoto et al. [33] 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 [89, 90]. Nevertheless, 21U-RNAs are regulated by fork-head transcription factors [91] and the fork-head factor FoxP1 regulates development in concert with SMRT [92]. 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 [93, 94] and organogenesis [95].

6.3 ALKB-8 2-oxoglutarate and Fe2+ 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 [96] 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.

6.4 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 [97].

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 [62] (Fedeles, Singh et al. 2015). ALKBH8 is known to regulate the rate of translation of thioredoxin reductase [80] which is one of the main enzymes important for dealing with oxidative stress [84, 98].

Our results as well as published data [70] 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 [70]. Our experiments as well as the data reported by Wormbase (WS263) [99] 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 [100] 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 [101]. In *C. elegans* (and in most sequenced animal species), there is another gene that is similar to AlkB8, that only has the methyl transferase domain, not the demethylase domain C35D10.12 (NP_497751.1) but nothing is known about its function.

7 CONCLUSIONS AND EVALUATION OF GOALS AND HYPOTHESES

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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List of publications

Publications related to thesis:

- 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)

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- 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.

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Publications not related to thesis:

- Kouns NA, Nakielna J, Behensky F, Krause MW, Kostrouch Z, Kostrouchova M. (2011) NHR-23 dependent collagen and hedgehog-related genes required for molting. *Biochem Biophys Res Commun* , 413(4):515-20.

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