Charles University
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Summary of Ph.D. Thesis

Regulation of gene expression and biological properties of cells: the potential of Mediator complex subunit 28

Regulace genové exprese a biologického chování buněk: potenciál podjednotky 28 Mediátorového komplexu

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Contents

ABSTRACT

The aim of the present study was to explore whether the dual localization and function of the MED28 orthologue is also conserved in nematodes and to characterize its regulatory potential. The work showed that the protein denominated in databases as the orthologue of MED28 (W01A8.1) is in fact the sole orthologue of mammalian perilipins and identified the real MED28 orthologue in the *C. elegans* genome (F28F8.5), that was renamed MDT-28 with the approval from WormBase. MDT-28 was shown to be indispensable for several developmental and growth processes in *C. elegans*. It has, similarly as in mammals, a dual cytoplasmic and nuclear localization. Using bioinformatic tools, MDT-28 was identified as a likely target for lysine acetylation and this was then proven experimentally. Further, valproic acid, a known inhibitor of lysine deacetylases, increased MDT-28 acetylation and decreased MDT-28 nuclear localization, suggesting that the nuclear localization of MDT-28 can be regulated. The results indicate that the orthologues of MED28 have the evolutionarily conserved potential to integrate regulatory signals from cytoplasmic structural proteins with the regulation of gene expression at the level of the Mediator complex and suggest new roles of regulated lysine acetylation at the level of the Mediator.

ABSTRAKT

V modelovém systému *C. elegans* bylo zjišťováno zda ortolog Mediátorové podjednotky MED28 je součástí genomu hlístic a zda je jeho duální lokalizace a funkce zachována také u těchto organismů s cílem dále charakterizovat jeho regulační potenciál. Práce prokázala, že protein původně označený v bioinformatických databázích jako ortolog MED28, v nomenklatuře *C. elegans* MDT-28, je ve skutečnosti jediný ortolog savčího proteinu perilipinu. Dále bylo zjištěno, že skutečný ortolog MED28 u hlístic je identický s dříve necharakterizovaným hypotetickým proteinem F28F8.5, který byl na základě této práce překlasifikován jako skutečný MDT-28. Bylo prokázáno, že F28F8.5 (MDT-28) je nezbytný pro četné vývojové a růstové procesy a má, podobně jako savčí MED28, duální jadernou a cytoplasmatickou lokalizaci. MDT-28 byl pomocí bioinformatických nástrojů identifikován jako cíl pro acetylaci lysinů, což bylo prokázáno i experimentálně. Valproová kyselina, která je známým inhibitorem deacetyláz histonů, zvyšovala úroveň acetylace MDT-28 a snižovala jadernou lokalizaci MDT-28, což ukazuje, že jaderná lokalizace MDT-28 je regulovatelná. Získané výsledky naznačují, že ortology MED28 mají evolučně zachovalý potenciál integrovat regulační signály z cytoplasmatických strukturních proteinů s regulací genové exprese na úrovni Mediátorového komplexu, a navrhují nové role regulované acetylace lysinů na úrovni Mediátoru.

1. INTRODUCTION

1.1 The Mediator complex

The Mediator complex or Mediator is a multisubunit protein complex that emerged from studies done over the past 25 years focused on unraveling the molecular mechanisms of gene expression regulation. Mediator is a critical structure transmitting the regulatory potential of transcription factors (TFs) towards the basal transcriptional machinery. In addition, this complex integrates numerous cell regulatory cascades with the regulation of gene expression [1]. This complex consists of 25 subunits in yeast and 30 subunits in mammals [1]. The Mediator subunits are conserved in-between species and clear orthologues of the majority of subunits can be identified in evolutionarily distant species [2-4]. The sequence conservation is however very low and the similarity between orthologues is overall quite little making the clear identification of some orthologues difficult [5, 6].

The Mediator subunits can be divided into those that are evolutionarily old, present in both unicellular as well as in multicellular eukaryotes and subunits that are considered more evolutionarily new and which are found only in multicellular organisms, more specifically in Metazoa. The later group consists of MED23, MED25, MED26, MED28 and MED30 [1, 4, 7, 8].

Mediator has a modular structure with four recognizable higher structures referred to as head, middle, tail and kinase modules [4]. The evolutionarily "new" subunits are found in three of the four modular structures as follows: MED28 and MED30 in the head module, MED26 in the middle module and MED23 and MED25 in the tail module.

1.2 Mediator complex subunit 28

Mediator complex subunit 28 (MED28) was originally found as a gene expressed predominantly in endothelial cells and named Endothelial-derived Gene 1 (EG-1) [9]. This protein was later renamed Magicin (merlin and Grb2-interacting cytoskeletal protein) and shown to be a cytoplasmic protein associated with the cell cytoskeleton and interacting with Grb2 and Merlin (a.k.a. Neurofibromin 2 or Schwannomin) [10]. In addition, it has been found to be a substrate for several Src-family kinases [11]. This protein was independently recognized as a Mediator complex subunit and renamed MED28 [12, 13] and was shown to regulate development of mesenchymal cells as a negative regulator of differentiation of progenitor cells into smooth muscle cells in cell culture [12]. The dual role and dual localization (nuclear and cytoplasmic) suggest that MED28 may connect regulatory events localized at the cell cytoskeleton with regulation of gene expression at the core of transcription [11]. Taken together, MED28 represents a protein that may have the potential to connect cytoplasmic events linked to cell differentiation states with the regulation of gene expression at the level of the Mediator complex and a very appealing protein for developmental studies.

1.3 Perilipins

Perilipins make up a family of proteins found at the surface of lipid droplets (LD). LDs are lipid storage organelles found in the cytoplasm of almost all cell types in mammals [14] as well as in insects, Dictyostelium, yeast, plants and some prokaryotes [15]. The structure of LDs comprises a hydrophobic core made from a stored neutral lipid (triacylglycerides or sterole esters such as cholesteryl esters) surrounded by a monolayer of phospholipids and a wide variety of proteins, the most abundant of which are the perilipins [14-16].

1.4 Acetylation

Acetylation can be classified into two broad categories, N-terminal acetylation (Nt-acetylation, N-α-acetylation), which falls outside of the scope of this thesis and acetylation of lysine in a polypeptide chain (N-ε-lysine acetylation or lysine acetylation).

From its discovery on histone proteins in the 1960s [17, 18], lysine acetylation is a posttranslational modification (PTM) which has been gaining more and more attention for its many regulatory roles in a variety of biological processes and its importance is comparable to that of phosphorylation. Just like phosphorylation it is evolutionarily conserved and in lower organisms the degree of conservation of the acetylome even surpasses that of the phosphoproteome [19]. Lysine acetylation is reversible and dynamic, the result of counteracting activities of histone acetyltransferases or lysine acetyltransferases (HATs a.k.a. KATs) and deacetylases. In 2007 HATs were renamed lysine acetyltransferases (KATs) to point out the broad substrate specificity which includes many different proteins not just the histones [20].

Lysine/histone acetylation is rapidly and effectively removed by histone/lysine deacetylases (HDACs/ KDACs) that are divided into class I, class II, class III (sirtuins), and class IV which contains only one representative HDAC11. While class I, II and IV are Zn^{2+} dependent aminohydrolases, class III HDACs' use NAD^{+} as co-substrate for their catalytic activities [18].

Prime targets of lysine reversible acetylation are proteins involved in cellular mechanisms that respond quickly to changing metabolic and other physiological conditions. This includes numerous events connected to the regulation of gene expression. Association of lysine acetylation with the level of gene expression was noticed in yeast [21] and it has been also proposed as the mechanism underlying regulation of gene expression by transcription cofactor GCN5 [22]. The discovery of nuclear receptor co-activators, namely CBP and p300 extended the possibility of acetylation of histones connected with the regulation of gene expression to other regulatory complexes (reviewed in [23]).

1.5 Inhibitors of lysine acetylation

Deacetylases as well as acetyltransferases can be effectively targeted by therapeutic interventions [24-26]. The activities of the aforementioned enzymes have been the focus of pharmacological modulators known as histone/ lysine deacetylase inhibitors (HDACIs KDACIs), which make up a rapidly expanding and broad group of substances. These compounds are both natural and synthetic, and have been gaining attention due to their promising role in anticancer therapy, mainly because of their epigenetic effects. They can be divided into several classes based on their structural composition, mainly: hydroxamic acids (e.g. suberoylanilide hydroxamic acid (SAHA) also known as vorinostat, trichostatin A), short chain fatty (aliphatic) acids (e.g. sodium butyrate, valproic acid), benzamides (e.g. entinostat) and cyclic tetrapeptides (e.g. depsipeptide) [24, 27]. HDACIs/ KDACIs also differ in their specificity of action and can be categorized into those that affect several classes of HDACs/ KDACs, known as pan-HDAC/ KDAC inhibitors (e.g. vorinostat, trichostatin A, valproic acid) and those that have a more specific inhibition, mainly affecting only one class of HDACs/ KDACs or a specific HDAC/ KDAC isoform [27-29].

1.6 Valproic acid

Valproic acid (2-propyl pentanoic acid or n-dipropylacetic acid, VPA) is a small branched fatty acid first synthesized in the 1880s as an analogue of valeric acid (naturally produced in the plant *Valeriana officinalis*) by B.S. Burton, who studied organic solvents, and

later found to have anti-epileptic properties by P. Eymard and his colleagues [24, 30]. It is a FDA-approved therapeutic indicated in the treatment of epilepsy (of absence and complex partial seizures), mania in bipolar disorder, as prophylaxis against migraine headaches, and in the treatment of certain cancers (e.g. leukemia and some solid tumors), either as monotherapy or in combinatorial drug regiments, or as a radiosensitizing agent [31-34].

1.7 Protein complexes that execute nuclear functions

Protein complexes are constantly disassembled and assembled but may exist as preformed modules. Components of the preinitiation complex (PIC) are recruited on the promoters of regulated genes in a stepwise manner [35]. Mediator is disassembled from promoters following the phosphorylation of the carboxy-terminal domain of polymerase II by TFIIH [36]. If taking into account the numerous situations of viable yeast cells despite being deficient for individual yeast Mediator subunits it can be assumed that the Mediator complex can also be disassembled into individual subunits or preformed Mediator modules or more likely sub-complexes.

Proteomic analyses identified more than 2700 mammalian protein complexes that show the tendency of association of small proteins with relatively big proteins based on domain-domain interactions [37]. Protein complexes show tendency of sharing functionally linked proteins and association with numerous to date uncharacterized proteins that are likely to contribute to specific roles of individual complexes [38, 39].

Protein interactions are regulated by PTM [40]. Protein-protein interactions are frequently functionally connected [40]. More than 60 % of PTMs are found in regions that are engaged in specific protein-protein interactions [41]. Lysine acetylation is one of the most frequent PTMs found in mammals [41].

1.8 Intrinsically disordered regions are critical for versatile protein interactions

Informatics and the wealth of structural data deposited into databases enhanced understanding of protein structures. In addition to fundamental building blocks of proteins consisting of alpha helices and beta sheets, additional structural fragments were recognized including loops, bends and other motifs found in structurally studied proteins. These motifs are deposited in numerous structural databases and allow bioinformatics searches across stored data (e.g. MegaMotifBase (http://caps.ncbs.res.in/MegaMotifbase/index.html) including 1032 protein structure families and 1194 superfamiles) [42]. Beside that, many proteins show lack of detectable structural motifs and contain regions denominated as proteins with low structural complexity, low globularity, intrinsically unstructured proteins or unfolded proteins [43]. These proteins showing signs of intrinsic structural disorder are now in the focus of biological studies.

1.9 *Caenorhabditis elegans* is a suitable model organism for research on the regulation of gene expression

The model system *Caenorhabditis elegans* is an efficient tool for studies aimed at the elucidation of the regulatory potential of MED28 in the regulation of development and cell differentiation.

C. elegans contributed importantly to the modern biological research in multiple ways. Following the proposition by Sydney Brenner to use *C. elegans* as a laboratory tool for biological research in 1973 [44], this system allowed for many discoveries such as the conserved developmental regulatory pathways, the basal mechanism of programmed cell death and *in vivo* tracking of proteins fused to the Green Fluorescent Protein (GFP) and its derivatives [45].

2. THESIS HYPOTHESIS AND GOALS

The goal of this thesis was to study an evolutionarily conserved protein whose function would be both nuclear and cytoplasmic and to find out if it would be regulated by epigenetic modification. Our bioinformatic analyses indicated a possible misclassification of our protein of choice, Mediator complex subunit 28, in the model system *C. elegans*. Based on these data the hypotheses were as follows:

- The protein F28F8.5 could be the MED28 homologue in nematodes, rather than the established W01A8.1. If the true orthologue of MED28 does exist in the *C. elegans* genome but is yet unrecognized, it would likely have conserved features with MED28 in its phenotypic and functionally conserved features as well in its dual cytoplasmic and nuclear localization. This intracellular localization is likely be regulatable.
- On the contrary, the protein W01A8.1 denominated in databases as Mediator complex subunit 28 is more likely an orthologue of perilipins believed not to exist in nematodes.

The aims of this thesis were:

- To find the closest possible homologue of mammalian MED28 in the *C. elegans* genome and then to test its in vivo effects in the model system *C. elegans* using the latest scientific methods.
- The next aim was to use valproic acid as an acetylation tool to test its effect on the already proven *C. elegans* MED28 orthologue.

3. METHODS

3.1 Sequence analyses

To analyze the nucleotide and protein sequences of the studied proteins (perilipin orthologues, W01A8.1, MED28 orthologues, F28F8.5) the NCBI (ncbi.nlm.nih.gov), UniProtKB (uniprot.org) and OMA (omabrowser.org) databases were used together with the programs BLAST, PSI-BLAST, HHblits and HHpred to retrieve and align the sequences along with the T-Coffee algorithm or PROMALS. To view and analyze the sequences Jalview was used. The programs PSIPRED, PAIL and GPS-PAIL enabled us to predict the secondary structures, lysine acetylation on internal lysines and the substrates of 7 HATs/KATs, respectively, as described [46-48].

3.2 Strains, transgenic lines and genome editing

For our work, we used either the wild type Bristol N2 strain wherever not otherwise specified or one of the following transgenic lines:

1. *C. elegans* transgenic lines made using CRISPR/Cas9 system:

1a. *C. elegans* transgenic line expressing W01A8.1 isoforms a, b or c tagged by GFP under *W01A8.1* natural promoter; **W01A8.1a/c::GFP** and **W01A8.1b::GFP** as described [46].

1b. *C. elegans* transgenic line KV3 (8418) propagated as a heterozygous line; one allele coding for ***F28F8.5 (V:15573749)::gfp::let858(stop)::SEC::F28F8.5*** (this allele has edited *F28F8.5* disrupted by *gfp* and SEC, allele is named "edited disrupted *F28F8.5"*), which is deficient for F28F8.5 and one allele consisting of wild type *F28F8.5* [47]. This line gives segregated mutant animals in the Mendelian pattern.

1c. *C. elegans* transgenic line KV4 (8419) which is a homozygous line; each allele contains $\boldsymbol{P_{F28F8.5}}$***(V:15573749)::gfp::F28F8.5*** (this allele has edited *F28F8.5* with *gfp* tagged to the N-terminus in the position *V:15573749*, allele is named "edited *gfp*::*F28F8.5*") [47].

2. *C. elegans* transgenic lines carrying extrachromosomal arrays:
2a. *C. elegans* transgenic line expressing human PLIN1, PLIN2, PLIN3 tagged by GFP under *W01A8.1* natural promoter: **PLIN1::GFP**, **PLIN2::GFP**, **PLIN3::GFP** [46].
2b. *C. elegans* transgenic line **F28F8.5::GFP** containing $P_{F28F8.5(400\ bp)}$***::F28F8.5::gfp*** and expressing *C. elegans* F28F8.5 tagged at its C-terminus with GFP and regulated by the *F28F8.5* natural promoter with the size of 400 bp upstream of the ATG [47].
2c. *C. elegans* transgenic line expressing GFP alone under *nhr-180* promoter. This line was used for control of GFP::MDT28 pull-down experiments [48].

Genome editing

For preparation of lines expressing the wild type F28F8.5 or W01A8.1 tagged with GFP on their N- and C-terminus, respectively, the CRISPR/Cas9 system was used as developed and described by [49-52] with modifications [46, 47].

For gene editing of *F28F8.5* to the form $P_{F28F8.5}$*::gfp::F28F8.5* (GFP is at the N-terminus of the protein), the endogenous gene was edited by insertion of a construct containing the coding sequence of GFP, the self-excising cassette (SEC), which includes the *sqt-1(d)* gene (inducing the Rol phenotype used as a selection marker of successful microinjection and transfer to the progeny), *hs::Cre* (heat shock inducible Cre recombinase) and *hygR* (hygromycin resistance) genes. The detailed description of the technique is provided in supplementary information connected to the primary publication by [47].

A similar strategy was used for the editing of *W01A8.1* to the form $P_{W01A8.1}$*::W01A8.1::gfp* (GFP is at the C-terminus of the protein) and for the preparation of W01A8.1 null mutant by the CRISPR/Cas9 system. Since this strategy leads to the insertion of GFP to the C-terminus, the expression of mRNA splice forms a and c (that have identical 3' ends) should proceed in the natural or close to natural ratios. This allowed the preparation of the following lines: W01A8.1a/c::GFP and W01A8.1b::GFP. The detailed description of the technique is provided in supplementary information connected to the primary publication [46].

3.3 General biochemical and molecular biology methods

The methods used for the experimental work forming the basis of this thesis included molecular biology methods of the model system *C. elegans* and standard biochemical methods and included: isolation of total RNA and genomic DNA from nematode populations, synchronized *C. elegans* cultures as well as individual animals of wild type (N2) and specific *C. elegans* strains; preparation of complementary DNA (cDNA) and subcloning into bacterial as well as eukaryotic vectors, RNA interference (RNAi), fecundity and brood size assays, PCR including quantitative PCR, microinjections for preparation of transgenic lines and for dowregulation of targeted genes by injection of dsRNA into the ovarial syncytium of parent animals. The details of targeted sequences, specific primers and other experimental details can be found in the method sections of the attached publications.

3.4 Microscopy and image analyses

Fluorescence microscopy and **Nomarski optics microscopy** were done using an Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). Animals were analyzed on microscopic glass slides with a thin layer of 2% agarose and immobilized by 1mM levamisole (Sigma-Aldrich, St. Louis, MO, USA).

Confocal microscopy and fluorescence lifetime imaging microscopy (FLIM) was done using an inverted Leica SP8 TCS SMD FLIM system equipped with acousto-optical excitation control (AOTF) and acousto-excitation emission separation (AOBS) (which allow

free tunability and high speed switching), two internal hybrid single photon counting detectors and a pulsed white light laser (470-670 nm) operating at femtosecond mode. The system was operated by Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany). For optical acquisition a 63x 1.2 NA water immersion objective was used.

Coherent anti-Stokes Raman scattering microscopy (CARS) was performed by Dr. Ahmed Chughtai in Leica Microsystems research and development facility in Mannheim, Germany with the kind help of Dr. Zhongxiang Jiang as described [46]. **Image analyses were done using** using the ImageJ computer program available from ImageJ website with appropriate plug-ins (http://imagej.nih.gov/ij/).

3.5 Binding studies

In order to prove the possible involvement of F28F8.5 as a subunit of Mediator, we searched for physical interactions of F28F8.5 with known Mediator subunits using two strategies. The first strategy included radioactively labeled proteins which were prepared using a coupled *in vitro* transcription and translation system (Promega, Madison, WI, USA) together with ^{35}S Methionine (Institute of Isotopes, Budapest, Hungary). The second strategy consisted of bacterial expression of proteins with GST sequence used for pull-down experiments and detection of binding partners fused with FLAG sequence and detected by anti-FLAG antibody on Western blots.

3.6 GFP-Trap and proteomic analyses of MDT-28 acetylation

The level of GFP::F28F8.5 expression and its level of lysine acetylation was done using the GFP-Trap®_MA system (ChromoTek Inc., Hauppauge, NY, USA).

4. RESULTS

4.1 The hypothetical protein F28F8.5 is the nematode orthologue of Mediator complex subunit 28 and is a critical regulator of *C. elegans* development (Kostrouchova et al. (2017))

Identification of the closest homologue of vertebrate Mediator complex subunit 28 in *C. elegans*

A search in protein databases for a nematode homologue of mammalian MED28 using human MED28 (UniProtKB - Q9H204, MED28_HUMAN) did not identify any close homologue in *C. elegans* and other nematode genomes. However, profile-to-profile algorithms HHblitz and HHpred indicated highly significant relationship to an uncharacterized predicted *C. elegans* protein with cosmid denomination F28F8.5. WormBase WS248 listed two expressed mRNA isoforms from this gene, coding for proteins with the length of 200 and 202 amino acids. Searches using pre-aligned vertebrate and insect MED28 homologues retrieved F28F8.5 in three iterations with E< 10^{-48} and the probability of true positivity >99.99%). This contrasted with the results obtained for alignment of MED28 mammalian orthologues with the protein with cosmid name W01A8.1 that was denominated in the *C. elegans* database as the MED28 orthologue. Unlike F28F8.5, W01A8.1 showed only very limited homology in its C-terminal region.

This led us to a conclusion that F28F8.5 is more likely the closest homologue or indeed an orthologue of MED28. A PSI-BLAST analysis of selected Metazoan MED28 orthologues reveals variable N- and C-termini of 3–80 amino acids showing no conservation across metazoan phyla. The alignment shows that only the central core of about 110 amino acids is preserved in metazoan evolution.

Contrary to W01A8.1 which is relatively large (isoforms a, b and c, with the size of 415, 385 and 418 amino acids, respectively), MED28 has only 178 amino acids. The predicted protein translated from F28F8.5 thus has a similar size as mammalian MED28 orthologues. Moreover, PSI-BLAST indicated with high statistical probability that F28F8.5 is the only MED28 orthologue present in the *C. elegans* genome. The alignment of the central part of the amino acid sequences of MED28 homologues reveals conservation of folding into three helices.

F28F8.5 is a nuclear as well as a cytoplasmic protein

The gene expression of F28F8.5 was tested using two techniques: the extrachromosomal array technique and the CRISPR/Cas9 system. These techniques enabled us to prepare F28F8.5 labeled on the N-terminus or C-terminus with GFP. The localization of the modified F28F8.5 was analyzed using confocal microscopy and FLIM. F28F8.5 was detected in both the nucleus as well as the cytoplasm in most if not all cells from embryos into adulthood. However, the nuclear expression of GFP-tagged F28F8.5 was predominant.

F28F8.5 regulates development

To achieve loss-of-function, RNAi was used to down-regulate *F28F8.5* expression. Analysis of 2567 progeny of 17 young adult hermaphrodites inhibited for *F28F8.5* function by microinjection of dsRNA into the syncytial gonad revealed that F28F8.5 was essential for proper development. From the total progeny, 1127 animals were affected (44 %) exhibiting embryonic and larval arrest and a range of less severe phenotypes, including defective molting, protruding vulva that often bursts, male tail ray defects, and uncoordinated (Unc) movement.

Because the CRISPR/Cas9 technique initially disrupts the F28F8.5 gene on either one or both alleles, we were able to observe the effect of loss of function of F28F8.5 in vivo and detected sterility, among other phenotypes, in homozygous mutants created in this way, indicating F28F8.5 to be an essential gene.

Complete loss of F28F8.5 that occurred in homozygous animals with both disrupted alleles of F28F8.5 gene (that are found among the progeny of heterozygous animals carrying one disrupted allele and one edited allele *gfp::F28F8.5*) resulted in sterility. However, these animals were able to develop to adults. This demonstrates that a single maternal allele provides sufficient gene product to get homozygous null animals through much of development, but was insufficient for proper somatic and germline gonad development. The gonad was irregular, contained masses of undifferentiated tissue and showed problems of directional growth. Defective vulva formation was also observed.

F28F8.5 interacts with Mediator complex subunits

We also looked for the interaction between F28F8.5 and other Mediator complex subunits. The interaction was found between GST-labeled F28F8.5 and radioactively labeled MDT-6 or FLAG-tagged MDT-30, proving that F28F8.5 possesses biding properties expected from a MED28 orthologue.

4.2 Valproic acid decreases the nuclear localization of MDT-28 – the intracellular localization of MDT-28 is regulatable (Kostrouchova et al. (2018))

Bioinformatic analyses of MED28 orthologues reveal a high probability of lysine acetylation. To support our concept of a dual regulatory function of MED28 orthologues, we tested whether MDT-28 could relocate between the nucleus and the cytoplasm by changing its acetylation status. Bioinformatic analysis suggested that MDT-28 contains lysines which can be acetylated. Using the bioinformatic tools PAIL and GPS-PAIL, we analysed the amino acid

sequences of MED28 orthologues and found the most C-terminal lysines as possible targets of acetylation of the HAT CBP.

***C. elegans* GFP::MDT-28 is dynamically acetylated**

We then proceeded experimentally and used the *C. elegans* line KV4, we prepared earlier, expressing GFP::MDT-28 from its 2 alleles. GFP::MDT-28 was extracted using the TRAP system, which binds the modified protein by its GFP tag and detected lysine acetylation using a commercial monoclonal antibody. To enhance the detection of lysine acetylation we incubated the KV4 line with VPA and saw a positive increase in signal on Western blots. Even with the increase in detection the incubation times were much longer than the simultaneous detection of GFP::MDT-28 using a commercial anti-GFP antibody. Lysine acetylation may thus be in only a minority of GFP::MDT-28 molecules and may represent a protein state which rapidly changes.

Valproic acid decreases the intranuclear localization of GFP::MDT-28

In vivo on the subcellular level GFP::MDT-28 was detected using FLIM in nuclei of enterocytes and with a dotted pattern. After treatment with VPA the nuclear signal of GFP::MDT-28 decreased, perhaps pointing to a possible transfer of MDT-28 molecules into the cytoplasm or its degradation.

Detection of intrinsically disordered regions in MDT-28

We searched if intrinsically disordered regions can be detected by GlobPlot (http://globplot.embl.de/cgiDict.py) are conserved in MDT-28. As expected, the bioinformatics tool detects intrinsically disordered regions in the N-terminal part of MDT-28 as well as human MED28 and a globular domain in the second part of both proteins confirming the close relationship of F28F8.5 to MED28 and contrasting with W01A8.1 which lacks the corresponding IDR (Nagulapalli et al. 2016 and our own searches).

4.3 The protein originally denominated as the nematode orthologue of MED28 is the sole nematode orthologue of perilipin genes and regulates lipid metabolism in *C. elegans* (Chughtai et al. (2015))

C. elegans genome contains only one orthologue of MED28 – reclassification of W01A8.1 as the sole orthologue of mammalian perilipins (Chughtai et al. (2015)).

Inspection of W01A8.1 that was originally classified as the nematode orthologue of MED28 suggested gross differences in comparison to all known MED28 orthologues.

Firstly, W01A8.1 is approximately two times bigger than the mammalian MED28. Secondly, even though some similarity between W01A8.1 and MED 28 was recognized by BLAST in their C-terminal parts, the overall similarity was very low. Keeping with this, when using a more sophisticated Position-Specific Iterative BLAST (PSI-BLAST), which is able to identify distant evolutionary relationships of proteins with lower sequence conservation, identified W01A8.1 as a possible homologue of human perilipins. The sequence alignment of the 3 main domains that are characteristic for perilipins: N-terminal PAT domain, imperfect amphiphilic 11-mer repeat and the C-terminal four-helix bundle.

On the protein level, the localization of human perilipins and W01A8.1 protein products both localized in the cytoplasm on vesicular structures identified as lipid droplets. This was concluded based on the following experiments. First, translational fusion constructs *W01A8.1b::gfp* and *W01A8.1a/c::gfp* localized on cytoplasmic vesicular structures that were

positive for LipidTox staining, confirming lipid droplets as shown for Drosophila PLIN1::GFP and human PLIN1::GFP and PLIN2::GFP in *C. elegans* lines expressing these proteins. Secondly, knockdown or knockout experiments pointed at lipid containing structures as the main target of W01A8.1 function.

After RNAi of W01A8.1, using both the feeding method and the microinjection method, the number of progeny was detected to be decreased by approximately 30%. Morphometric analysis of CARS microscopy pictures of W01A8.1 null embryos and adult animals observed more CARS positive signal in embryos and less signal in adults compared to control animals indicating the presence of more fat in embryos and less amount of fat in adults. Also the size of the lipid containing structures differed; in null embryos vs control embryos these structures were larger, while in the somatic cells of larvae and adult inhibited animals these structures were smaller and more numerous.

Most importantly concerning the classification of W01A8.1 as the MED28 orthologue, our searches did not prove a pronounced nuclear localization of W01A8.1.

5. DISCUSSION

5.1 Conserved characteristics of MED28 orthologues

Conserved features of MED28 orthologues in mammals and in nematodes

The orthologue of the mammalian Mediator subunit 28 does exist in nematodes (predicted hypothetical protein F28F8.5) [47] but it is not the previously denominated protein W01A8.1 [2] which is in fact the sole orthologue of mammalian perilipins [46]. The proper classification of both W01A8.1 and F28F8.5 has important consequences. While the incorrect classification of W01A8.1 led to the false interpretation that perilipin-dependent regulation of lipid metabolism is not present in nematodes [53] [54], the classification of the perilipin orthologue as a MED28 orthologue suggested that the nematode MED28 orthologue was substantially different from its mammalian counterparts. Our findings support re-classification of W01A8.1 as the nematode orthologue of mammalian perilipins and F28F8.5 as the nematode MED28 orthologue by several lines of evidence that were accepted by WormBase for the formal renaming of both proteins as PLIN-1 and MDT28, respectively.

As expected, nematode PLIN-1 (W01A8.1) with attached GFP labelled lipid-containing structures in the cytoplasm and was not detected at predominant levels in cell nuclei. PLIN-1 loss of function led to clear alteration of lipid containing structures [46]. In contrast, MDT-28 (F28F8.5) expressed as GFP tagged proteins from extrachromosomal arrays or from its endogenous gene edited to be expressed as a protein tagged on its N-terminus with GFP was found predominantly in the nucleus with cytoplasmic localization that was not on lipid-containing structures [47].

Conserved structural features of nematode and mammalian MED28 orthologues

The nematode and mammalian MED28 orthologues have almost identical size (200 and 202 amino acids for the nematode MDT-28 isoforms a and b; and 178 amino acids for human MED28). A high degree of similarity between nematode and human MED28 orthologues was revealed by PSI-BLAST. A search for the presence of intrinsically disordered regions (IDRs) and globular domains using the bioinformatic tool GlobPlot indicated that the N-terminal parts (with the length of approximately 90 amino acids) of both nematode and human MED28 orthologues have signs of being disordered sequences while the C-terminal portion of both proteins are globular [48].

IDRs are very common in most Mediator subunits and are likely to be very important for their functional plasticity. An increment in complexity can be seen when comparing the

number and size of IDRs in multicellular organisms versus that of yeast. They are also accompanied by an increasing likelihood of more complex posttranslational modifications in mammalian orthologues compared to yeast counterparts which is keeping with more complex protein-protein interactions that are expected to be necessary for regulations in multicellular organisms [55].

Conserved functional features - nematode and mammalian MED28 orthologues' interact with MDT-6 and MDT-30

Contrary to the majority of the Mediator subunits, MED28 does not have a yet completely clear localization within the Mediator structure [56]. It has been proposed to be part of the tail module, the head module or to be represented as a density between the head and middle modules. It was shown to be engaged in direct contacts with the head module subunits MED27, MED30, MED6 and MED8 [57].

Our experiments confirmed the physical interactions of MDT-28 with MDT-6 (prepared as a radioactively labelled protein made *in vitro* using a coupled transcription-translation system with rabbit reticulocyte lysate) and MDT-30 (prepared as a bacterially expressed protein) [47]. These interactions were much stronger than those studied using the bacterially expressed MDT-6 and *in vitro* expressed MDT-30. Taken together this not only validates F28F8.5 as a Mediator subunit and the true orthologue of MED28 but also suggests that the low capacity for certain PTMs to occur during heterologous protein expression may critically influence both the production of individual Mediator subunits as well as their studied interactions [47, 58, 59].

MED28 orthologues in nematodes and mammals share a dual nuclear as well as cytoplasmic localization

Most Mediator subunits show prominent nuclear localization as can be anticipated for transcriptional coregulators. MED28 has in this respect a special position which manifests as having both a nuclear as well as cytoplasmic localization and function. It has been originally recognized as a cytoplasmic protein connected to the cell cytoskeleton and interacting with NF2 and Grb2 proteins [10] and shown to be functioning as a regulator of MEK1-dependent cellular migration in human breast cancer cells [60]. Its proven interactions with cytoplasmic proteins suggest it may have a regulatory potential to connect cell structural states with the regulation of gene expression [11].

Besides MED28, other Mediator subunits have also been shown to possess some additional non-genomic functions. For example MED12 was shown to function in the cytoplasm where it directly blocks TGFb signaling by interacting with TGFbR2. This is likely to be connected with a decreased activity of MED12 in a subset of drug-resistant tumors [61]. The potential to interact with non-nuclear proteins may as well be expected for MED 8, 13, 20, 21, 23, 26 and 27 based on the reported extra nuclear localization seen in The Human Protein Atlas (https://www.proteinatlas.org, accessed on February 25, 2019).

Our findings of the conserved dual localization of the nematode and mammalian MED28 orthologues further supports the classification of F28F8.5 as the true orthologue of MED28. It also suggests that the dual nuclear as well as cytoplasmic localization is a property that is conserved between distant phyla.

MDT-28 has general as well as specific functions in the regulation of development

The knowledge that F28F8.5 is the nematode orthologue of MED28 makes possible to combine data mining from information available for predicted genes from *C. elegans* high

throughput experiments and other functional data deposited to WormBase together with phenotypes of loss of function and downregulation of F28F8.5 that we obtained in our experiments. The observed phenotypes also support the dual roles of F28F8.5.

The observed phenotypes induced by F28F8.5 downregulation by RNAi included general defects of embryonic and larval development that can be expected to be seen when downregulating a major transcriptional cofactor, which should be the case for a constituent of the Mediator complex. They included arrests of embryonic and larval development at different stages. Additional phenotypes triggered by RNA interference indicated that F28F8.5 is critically involved not only in general transcription regulation but also in specific regulatory cascades that may be both genomic and non-genomic. The first types of phenotypes included defects of gonadogenesis, molting defects, atrophy and formation of defects in *C. elegans* tissues. The second class of phenotypes are likely to be connected with cell regulatory cascades, especially the EGFR cascade and include the developmental defects of the vulva and of male specific structures, the male rays [7, 62].

The developmental role of F28F8.5 may be evolutionarily conserved and seen in mammals as a connection of MED28 with Grb2. The closest nematode homologue and likely ortholgoue of Grb2 is SEM-5, which has a known role in the regulation of development of male rays. F28F8.5 protein contains a predicted SH2 binding site for Grb2 similarly as MED28, which can be identified using the site prediction informatics tool Motif Scan (http://scansite.mit.edu/motifscan_seq.phtml) [10]. Similarly, the burst through vulva phenotype is also likely to be connected with LET-60/Ras signaling [63, 64]. The conservation of the dual, nuclear and cytoplasmic functions of MED28 homologues is therefore likely to be present throughout the evolution of Metazoa.

Our data uncovered a striking difference between the complete elimination of MDT-28 (F28F8.5) and its downregulation by RNAi. In contrary to MDT-28 (F28F8.5) downregulation by RNAi, the animals lacking both alleles of F28F8.5 that were found in the progeny of heterozygous animals carrying one wild type F28F8.5 and one null allele were able to reach the L4 stage or adulthood but had an undeveloped gonad and were completely sterile. These phenotypes were less severe than some developmental defects seen after downregulation of MDT-28 (F28F8.5) expression by RNAi. There are several possibilities that may explain our findings. The most plausible explanation is the very different consequences of both methods on progeny development. While RNAi strongly inhibits gene expression in the gonads of hermaphrodites of all stages, both in the germline and in the spermatheca, as well as in their progeny exposed to the dsRNA, the progeny lacking both alleles of F28F8.5 (homozygous for the null alleles) found in mother hermaphrodites (heterozygous) are likely to receive an important amount of maternally delivered MDT-28 (F28F8.5). Since the total mass of fertilized oocytes and embryos up to the L1 stage is similar, the maternally supplied MDT-28 may be sufficient for a big part of the embryonic development. It may perhaps have effects even during early larval development when only a small number of new cells is developing. However, this possibility would require a long half-life of MED28. Another less likely possibility is genomic compensation which was suggested as an explanation to similar findings in the case of several genes, e.g. egfl7, a gene involved in the production of connective tissue in blood vessel walls. Experiments done in zebrafish showed that while the Egfl7 knockout animals had blood vessel growth unaffected, the knockdown of egfl7 led to abnormal blood vessel development. It was found that in knockout animals another gene, emilin 3B, was produced in higher amounts and was able to compensate for the function of egfl7 [65].

5.2 MDT-28 is a dynamically acetylated protein

Bioinformatic analysis of F28F8.5 reveals potential lysine-acetylated sites in the primary protein sequence and the presence of acetylated lysines was supported by immunodetection of acetylated lysines in GFP::F28F8.5 expressed from genome-edited homozygous animals and immunoprecipitated using the GFP-TRAP system [47]. The relative proportion of the positive signal detected by anti-acetylated lysine antibody was augmented in lysates from animals treated with VPA in comparison to the intensity of the signal produced by the anti-GFP antibody. This supports the specificity of the anti-acetylated lysine detection. It also suggests that lysine acetylation in MDT-28 is dynamic and is likely to be subjected to deacetylation by HDACs sensitive to VPA.

The bioinformatic detection of high probability of lysine acetylation of MDT-28 dependent on CBP can be expected in light of numerous identified non-histone target substrates and the presence of CBP/p300 at the core of transcription initiation (at the PIC complex). Our experiments suggest that the proportion of acetylated MDT-28 detected by the specific antibody may be small in comparison to the total GFP::MDT-28. However, it may also be the case that the antibody against acetylated lysines may have lower affinity towards the antigen, as was reported for several anti-lysine antibodies and are likely to be influenced by the composition of the site surrounding the acetylated lysine.

5.3 The intracellular localization of MDT-28 is sensitive to treatment with valproic acid

Our functional experiments support the effects of VPA on F28F8.5 at the biological level. The decreased presence of GFP::MDT-28 in nuclei of VPA treated animals compared to controls further supports the role of VPA in the regulation of transcription. There are several possible explanations of this VPA effect on MDT-28. Lysine acetylation is a very effective regulator of protein-protein interactions of cytoskeletal proteins and is expected to be a general mechanism also affecting transcription regulation.

6. CONCLUSION

Based on the published results the proteins F28F8.5 and W01A8.1 are correctly denominated in databases as MDT-28 (the MED28 orthologue in nematodes) and PLIN-1 (the sole nematode orthologue of perilipins), respectively. The work was done using the most up to date bioinformatic and functional methods. F28F8.5/ MDT-28 was shown to be the MED28 orthologue in nematodes on the evolutionary conserved features of MED28 orthologues which are: has dual, both nuclear as well cytoplasmic intracellular localization and its loss of function phenotypes support its gross function in the regulation of gene expression visualized as developmental defects. The physical interaction of MDT-28 with two other mediator subunits, MDT-6 and MDT-30, was confirmed. The possibility of MDT-28 being acetylated at lysine residues was first detected bioinformatically before being confirmed experimentally. We showed that lysine acetylation of MDT-28 is regulated and is augmented by treatment with a known inhibitor of histone deacetylases, valproic acid. We found that treatment with valproic acid leads to a decreased nuclear presence of MDT-28 which supports the dual and regulated intracellular localization of MDT-28 and the potential of MDT-28 to connect cytoplasmic events with the regulation of gene expression at the level of the Mediator.

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8. LIST OF PUBLICATIONS

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