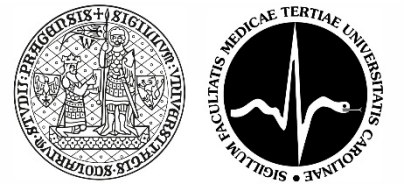

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MUDr. Markéta Kostrouchová

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

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

Ph.D. Thesis

Supervisor: prof. MUDr. Václav Mandys, CSc.

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CONTENTS:

1. ABBREVIATIONS

ATX3 – ataxin 3
BHC – a corepressor complex
bp – base pair
CARS – Coherent anti-Stokes Raman scattering
cDNA – complementary DNA
CREB – cAMP response element binding protein
CBP – CREB-binding protein, a.k.a. CREBBP
C. elegans – *Caenorhabditis elegans*
CRISPR/Cas9 – clustered regularly interspaced palindromic repeats/Cas9 system
DNA – deoxyribonucleic acid
DPY – dumpy phenotype
EGF – epidermal growth factor
Egfl7 – EGF-like domain 7
EGFR – epidermal growth factor receptor
F28F8.5 – gene coding for protein F28F8.5, ***F28F8.5a*** – splice form a, **F28F8.5a** – protein form a, ***F28F8.5b*** – splice form b, **F28F8.5b** – protein form b, **F28F8.5::GFP** – protein F28F8.5 tagged on its C-terminus with GFP, *F28F8.5* was renamed with permission from WormBase to MDT-28
FLAG – an octapeptide *sequence* of DYKDDDDK
FLIM – fluorescence lifetime imaging
GABA – gamma-aminobutyric acid
GCN5 – general control of amino acid synthesis 5, a.k.a. lysine acetyltransferase 2 (KAT2)
GFI – growth factor independence
GFP – green fluorescent protein, ***gfp*** – gene coding for GFP
GFP::F28F8.5 – protein F28F8.5 tagged on its N-terminus with GFP
Grb2 – growth factor receptor bound protein 2
GST – glutathione S-transferase
HAT/ KAT – histone acetyltransferase, a.k.a. lysine acetyltransferase
HDAC/ KDAC – histone deacetylase, a.k.a lysine deacetylase
HDACI/ KDACI - histone deacetylase inhibitor, a.k.a lysine deacetylase inhibitor
IDR, IDP – intrinsically disordered region, intrinsically disordered protein
IPTG – isopropyl β-D-1-thiogalactopyranoside
KDM1A – lysine demethylase 1A
L(1-4) stage – larval stage L1-L4
LET-60/RAS – let-60 is an orthologue of mammalian RAS GTP-ases
MED – Mediator complex subunit
MED28 – vertebrate Mediator complex subunit 28 gene, **MED28** – vertebrate Mediator complex subunit 28 protein, **Med28** – Mediator complex subunit 28 in a general sense; this nomenclature is also used in *Drosophila* and mouse gene nomenclature
MDT – nematode orthologues of MED proteins
MDT-28 (F28F8.5) – the nematode orthologue of MED28, F28F8.5 was renamed with permission from WormBase to MDT-28
MEK1 – a.k.a mitogen-activated protein kinase kinase 1 (MAP2K1)
mRNA – messenger RNA
N2 – *wild-type C. elegans* variety Bristol, strain N2
NAT – N-terminal acetyltransferase
NCBI – National Center for Biotechnology Information
N-CoR – nuclear receptor corepressor, a.k.a. NCOR1

NF2 – neurofibromin 2, a.k.a. merlin or schwannomin
NGM – nematode growth medium
NHR – nuclear hormone receptor, **NR(s)** – nuclear receptor(s)
NMDA – *N*-methyl-D-aspartate
OD – optical density
p53 – a.k.a. TP53 or tumor protein p53
PCR – polymerase chain reaction, **qPCR** – quantitative PCR
$P_{F28F8.5}$(V:15573749)::gfp::F28F8.5 – edited *F28F8.5* with *gfp* tagged to the N – terminus in the position *V:15573749*, allele named edited *gfp*::*F28F8.5*
$P_{F28F8.5}$(V:15573749)::gfp::let858(stop)::SEC::F28F8.5 – edited *F28F8.5* disrupted by *gfp* and SEC (self-excising cassette), allele named edited disrupted *F28F8.5*
$P_{F28F8.5(400\ bp)}$::F28F8.5::gfp - F28F8.5 tagged with GFP on its C-terminus regulated by its predicted internal promoter with the size of 400 bp upstream of the ATG
PIC – preinitiation complex
PLIN – perilipin
PLIN-1 (W01A8.1) – the nematode orthologue of perilipins, W01A8.1 was renamed with permission from WormBase to PLIN-1
PolII – DNA-dependent RNA polymerase II
PolII CTD – carboxy-terminal domain of polymerase II
PTM – posttranslational modification
RCOR1 – REST corepressor 1
REST – RE1 Silencing Transcription Factor
RFC5 – replication factor C subunit 5
RNA – ribonucleic acid, **dsRNA** – double stranded RNA
RNAi – RNA interference
SAHA – suberoylanilide hydroxamic acid, a.k.a. vorinostat
SEC – self-excising cassette
SEM-5 – a homologue of *Grb2*
SH2 – Src homology type 2
SL – splice leader
SMRT– silencing mediator for retinoid or thyroid-hormone receptors, a.k.a. nuclear receptor corepressor 2 (NCOR2)
Src – member of Src family kinases
TF – transcription factor
TCoA – transcriptional coactivator
TCoF – transcription cofactor
TCoR – transcriptional corepressor
TGFb – transforming growth factor-beta
TGFbR2 – TGFb receptor type 2
UV – ultraviolet
VPA – valproic acid

2. INTRODUCTION

2.1 Biological behavior of cells and gene expression, a historical perspective

Biological behavior of cells, their metabolism, renewal, development and differentiation of cells in the case of multicellular organisms depends on specific gene expression. This gene expression is based on cell and tissue specific transcription factors (TFs), that bind to specific sequences in promoters of regulated genes with the contribution of cell and tissue specific transcription cofactors (TCoFs) and transmit the regulatory potential towards the basal transcriptional machinery and additional executory and regulatory mechanisms (Rue & Martinez Arias 2015; Bradner et al. 2017).

TCoFs include numerous proteins that were identified as proteins physically binding TFs or critically important for the regulatory potential of TFs and include proteins from different protein classes (Coutts & La Thangue 2006; Sommerfeld et al. 2011; Stampfel et al. 2015). Some such proteins proved to be evolutionarily conserved and despite their relatively low primary sequence conservation, it was possible to recognize that they are constituents of evolutionarily conserved protein complexes involved in the regulation of specific gene expression on several levels from the regulation of transcription initiation, transcription elongation, mRNA splicing to other downstream mechanisms in the direction of gene expression (Huang et al. 2015; Bradner et al. 2017).

An important contribution in gaining knowledge of the regulation of gene expression at the molecular level was achieved in studies focused on the identification of proteins interacting physically with TFs and transmitting their regulatory potential towards promoters of regulated genes. This led to the identification of powerful transcriptional coactivators CBP/ p300, highly similar paralogues with acetyltransferase activities (Arany et al. 1994; Lundblad et al. 1995; Chakravarti et al. 1996; Eckner 1996; Eckner et al. 1996; Yang et al. 1996; Puri et al. 1997). The fact that some nuclear receptors (NRs) function as transcriptional activators

(with bound activating ligand) or as transcriptional repressors (in the absence of ligand) while in both cases being present in the nuclei of cells allowed the identification of proteins binding these TFs, such as the corepressors SMRT (renamed as NCoR2; Chen & Evans 1995) and N-CoR (renamed as NCoR1; Horlein et al. 1995), which are both involved in deacetylation of nucleosomal histones although indirectly, by interaction with histone deacetylases. Subsequently, additional newly identified TCoAs and TCoRs extended to a large set of proteins with various molecular functions (Brown et al. 2000; Lee et al. 2000).

Mechanistically, the acyl- and mostly acetyl-transferase activity of several coactivators was linked to nucleosomal histones residing in the promoters of regulated genes that show elevated levels of acetylation of specific lysines, which is related to enhanced activity of these regulated genes and was broadened to the concept of PTM of histones often referred to as the histone code (Jenuwein & Allis 2001).

The histone code involves several types of modifications, starting with acetylation, methylation (Margueron et al. 2005) and followed by many other PTMs (including phosphorylation, ubiquitination/ ubiquitylation, sumoylation, biotinylation, glycosylation and additional acyl modifications such as propionylation, butyrylation, and crotonylation (Janssen et al. 2017).

It has been assumed that PTMs of histones affect the affinity between DNA and nucleosomes, which in the acetylated state may be more relaxed and thus prone to transcription activation regulatory events. This concept was however extended to the concept of a wider protein interactome at the level of gene promoters and direct activation of executive proteins involved in the regulation of gene expression (Walia et al. 1998; Eberharter & Becker 2002; Park et al. 2018). This concept further includes cell regulatory cascades involved in the regulation of gene expression whereby the proteins of these cascades also posttranslationally modify (often by phosphorylation) TFs and TCoFs and modulate their

primary regulatory potential which is in this way further super-regulated or modulated (Tootle & Rebay 2005; Filtz et al. 2014).

2.2 The Mediator complex and perilipins

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 (Sierecki 2018). This complex consists of 25 subunits in yeast and 30 subunits in mammals (Sierecki 2018). In plants, additional proteins associating with Mediator subunits were identified (Sundaravelpandian et al. 2013; Zhang et al. 2013; Hemsley et al. 2014; Samanta & Thakur 2015; Seguela-Arnaud et al. 2015; Cao et al. 2016; Kim et al. 2016; Yang et al. 2016; Malik et al. 2017). It has been proposed that the composition of Mediator complexes may differ among plant species (Samanta & Thakur 2017). The Mediator subunits are conserved in-between species and clear orthologues of the majority of subunits can be identified in evolutionarily distant species (Bourbon et al. 2004; Bourbon 2008; Yin & Wang 2014). The sequence conservation is however very low and the similarity between orthologues is overall quite little making the clear identification of some orthologues difficult (Poss et al. 2013; Allen & Taatjes 2015).

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 latter group consists of MED23, MED25, MED26, MED28 and MED30 (Sierecki 2018; Grants et al. 2015; Soutourina 2018; Yin & Wang 2014).

Mediator has a modular structure with four recognizable higher structures referred to as head, middle, tail and kinase modules (Yin & Wang 2014). 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.

The evolution of multicellular organisms is connected with the evolution of an increasing number of TFs (300 in yeast, 1600 in *C. elegans* and probably close to 3000 in mammals) and transcription cofactors, also called co-regulators (Vaquerizas et al. 2009). The complexity of Mediator in multicellular organisms is greater than in the case of unicellular organisms suggesting that the demands for a more precise regulation of gene expression is necessary for the development of multicellular organisms and specialized cell types. Although several Mediator subunits were studied in greater detail concerning their interaction with TFs, the regulatory roles of individual Mediator subunits have to be still determined especially from the view of the regulation of an organisms' development.

Mediator complex subunit 28

Mediator complex subunit 28 (MED28) was originally found as a gene expressed predominantly in endothelial cells and named Endothelial-driven Gene 1 (EG-1) (Liu et al. 2002). 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) (Wiederhold et al. 2004). In addition, it has been found to be a substrate for several Src-family kinases (Lee et al. 2006). This protein was independently recognized as a Mediator complex subunit and

renamed MED28 (Sato et al. 2004; Beyer et al. 2007) 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 (Beyer et al. 2007). 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 (Lee et al. 2006). 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.

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 (Sztalryd & Brasaemle 2017) as well as in insects, Dictyostelium, yeast, plants and some prokaryotes (Cermelli et al. 2006). The structure of LDs comprises a hydrophobic core made from a stored neutral lipid (triacylglycerides or sterol 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 (Waltermann & Steinbuchel 2005; Cermelli et al. 2006; Sztalryd & Brasaemle 2017).

The number of genes differs in different organisms. Mammals have 5 perilipin genes (Plin 1-5) while tetrapods have 2 genes (Plin 3 and Plin 4/5) and fish have 4 genes (Plin 1, Plin 2, Plin 3/4/5 and Plin 6) (Sztalryd & Brasaemle 2017). Perilipins have not been identified in bacteria and not until recently were they detected in yeast (Gao et al. 2017) and by our laboratory in *C. elegans*.

2.3 Posttranslational modifications and lysine acetylation

Posttranslational modifications

Posttranslational modifications (PTMs) are changes made to amino acid residues of proteins and increase the functional complexity of the proteins and of the genomes as well.

PTMs can be categorized according to the reversibility of the process and the type of modification made. Thus reversible PTMs include: chemical modifications (phosphorylation, acetylation, methylation, and redox-based modifications), polypeptide modifications (ubiquitination/ ubiquitylation, SUMOylation, and modifications by other ubiquitin-like polypeptides), and modifications by complex molecules (glycosylation, lipid attachement, AMPylation, ADP-ribosylation); irreversible PTMs are modifications of amino acids or of the polypeptide backbone and include deamidation, eliminylation, and cleavage by proteolysis (Spoel 2018).

Acetylation

Acetylation can be classified into two broad categories. The first is N-terminal acetylation (Nt-acetylation, N-α-acetylation), during which an acetyl moiety (CH_3CO) is covalently attached to the nitrogen of the free α-amino group of the amino acid residues found on the N-terminus of newly formed polypeptide chains but also elsewhere, and mainly regulates protein stability and intracellular distribution (Drazic et al. 2016). It is mediated by N-terminal acetyltransferases (NATs) which belong to six groups named NatA to NatF (Starheim et al. 2012; Drazic et al. 2016) and is considered to be irreversible because no N-terminal deacetylases have been found so far (Ree et al. 2018).

The other type of acetylation refers more specifically to ε-acetylation of the ε-amino group of lysine in a polypeptide chain (N-ε-acetyl-lysine is formed) and is generally known as lysine acetylation.

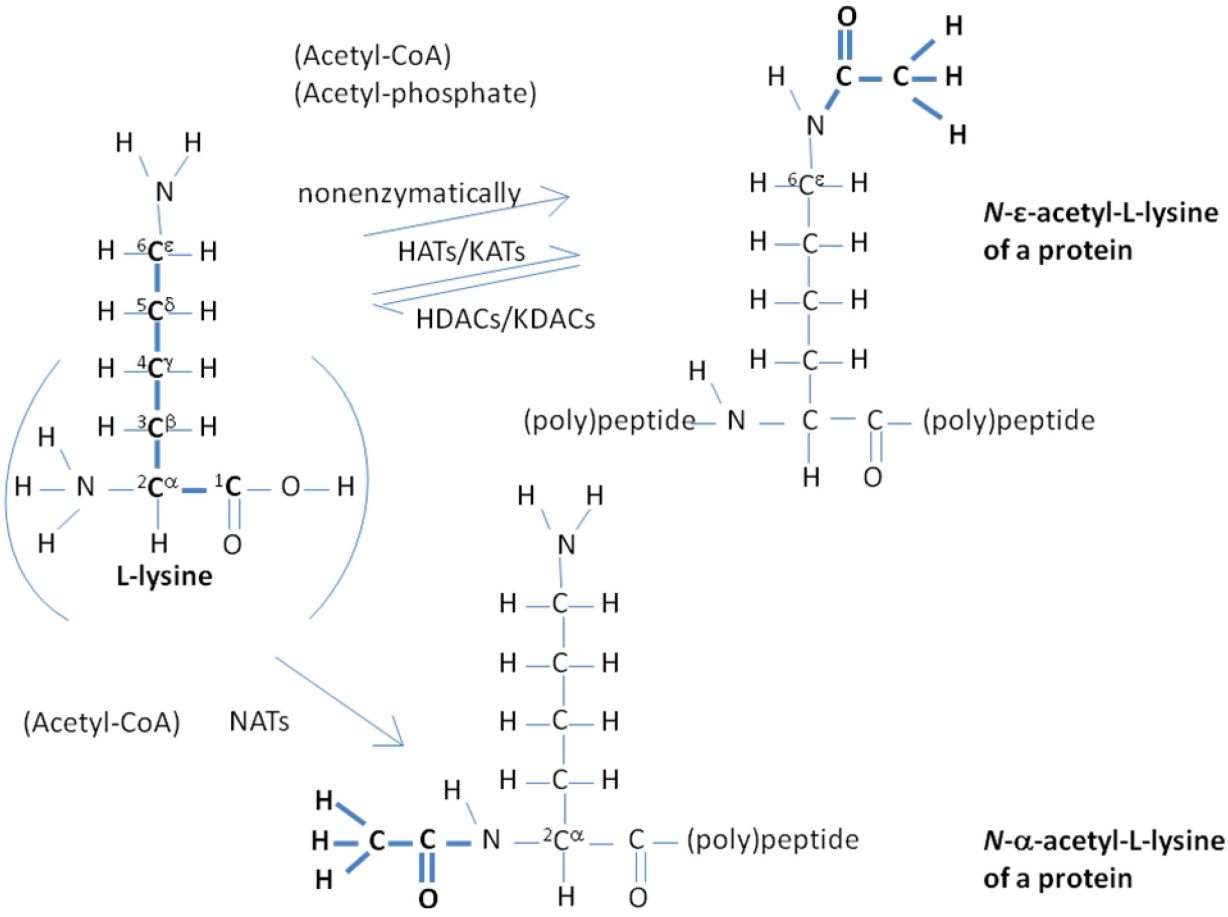

Figure 1. Schematic representation of types of acetylations in proteins (modified from (Drazic et al. 2016)).

From its discovery on histone proteins in the 1960s (Allfrey et al. 1964; Drazic et al. 2016), lysine acetylation, previously known as histone acetylation, 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 (Choudhary et al. 2009). 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 (Bedford et al. 2010).

HATs/KATs are usually classified as either type-A or type-B. Type-A include p300/CBP-like proteins, GNAT (GCN5) and MYST proteins (Bannister & Kouzarides 2011). Type-B HATs/ KATs acetylate newly formed H4 (at K5 and K15) as well as some lysines in H3 histone and are thought to act more like house-keeping genes than transcription regulators.

Lysine/histone acetylation is rapidly and effectively removed by lysine/ histone 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 (Drazic et al. 2016).

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 (Braunstein et al. 1993) and it has been also proposed as the mechanism underlying regulation of gene expression by transcription cofactor GCN5 (Brownell et al. 1996). The discovery of nuclear receptor coactivators, namely CBP and p300 extended the possibility of acetylation of histones connected with the regulation of gene expression to other regulatory complexes (reviewed in (Vo & Goodman 2001)). Although it has been clear that many diverse proteins may be substrates of acetyltransferases connected with gene expression activation, the first identified targets were histones. This later expanded to the concept of epigenetic gene expression regulation (the histone code). Histone modifications include primarily acetylation

and methylation of internal lysines and phosphorylation of serines, threonines and tyrosines (reviewed in (Bannister & Kouzarides 2011)).

Unlike phosphorylation that occurs mostly in relaxed protein regions, acetylated lysines are usually found in well-structured protein regions. The effect of acetylation of the ε-amino group is removal of the positive charge of the lysine's side-chain. This group can also be mono-, di- and tri-methylated (Bannister & Kouzarides 2011; Drazic et al. 2016).

Inhibitors of lysine acetylation

Deacetylases as well as acetyltransferases can be effectively targeted by therapeutic interventions (Kostrouchova et al. 2007; Scott 2012; Dancy & Cole 2015). 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) (Kostrouchova et al. 2007; Miller et al. 2011). 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 (Kim & Bae 2011; Miller et al. 2011; Van Dyke 2014).

Valproic acid

Valproic acid (2-propyl pentanoic acid or n-dipropylacetic, 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 (Burton 1882), who studied organic solvents, and later found to have anti-epileptic properties by P. Eymard and his colleagues (Meunier et al. 1963) (reviewed in (Kostrouchova et al. 2007; Rakitin 2017)). 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 (Kostrouchova & Kostrouch 2007; Chiu et al. 2013; Ochiai et al. 2016; Shah & Stonier 2019). Women of childbearing potential should not use VPA because of its known teratogenic effects, e.g. defects of neural tube closure such as spina bifida. Other serious negative effects include hepatotoxicity, hyperammonemic encephalopathy, pancreatitis, and thrombocytopenia. Just like its therapeutic use its action potential is also broad and still not fully elucidated. It was shown to increase the level of gamma-aminobutyric acid (GABA) concentration in the brain, to inhibit *N*-methyl-D-aspartate (NMDA) receptors, block voltage-dependent sodium channels and in 2001 was shown to possess HDAC inhibiting effects, to inhibit HDACs of class I and II (Kostrouchova & Kostrouch 2007; Shah & Stonier 2019).

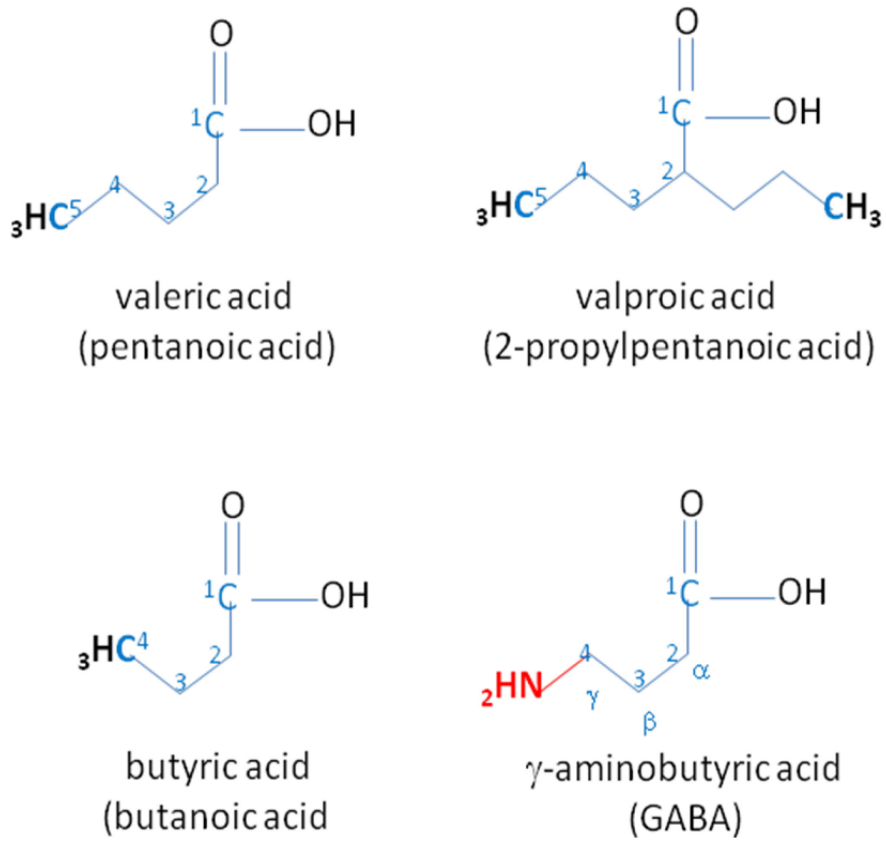

Figure 2. Schematic representation of valeric acid, valproic acid, butyric acid and gamma-aminobutyric acid (modified from (Kostrouchova & Kostrouch 2007; Rakitin 2017)).

2.4 Protein-protein interactions and intrinsically disordered regions

Protein complexes that execute nuclear functions

Protein complexes executing distinct nuclear molecular mechanisms related to Pol II dependent gene expression include **SAGA** (Spt-Ada-Gcn5 acetyltransferase) complex (Helmlinger 2012; Wang & Dent 2014; Helmlinger & Tora 2017), **ASAP** (Apoptosis- and Splicing-Associated Protein Complex; Deka & Singh 2017) **NMD** (nonsense-mediated mRNA decay) complex ((Schweingruber et al. 2016), **spliceosome complex** (Ohi et al. 2007; Finci et al. 2018). Gene expression is further regulated by higher organization of chromatin, which establishes genomic regions accessible or inaccessible to regulation by TFs, the transcriptional machinery and cooperating protein complexes. These complexes include the

chromatin remodeling complex **INO80** (Jin et al. 2005; Conaway & Conaway 2009; Tosi et al. 2013; Yao et al. 2016), **SWR1** containing complex (Mizuguchi et al. 2004; Zhou et al. 2010; Srivatsan et al. 2018), **SWI/SNF** (SWItch/Sucrose Non-Fermentable nucleosome remodeling complex) (Cote et al. 1994; Peterson et al. 1994; Dechassa et al. 2008; Sanz et al. 2012), **RSC** (Remodelling the Structure of Chromatin) complex (Cairns et al. 1996; Lorch et al. 1998; Huang et al. 2004), and the histone acetyltransferase **NuA4** complex (Wang et al. 2018; Klages-Mundt et al. 2018).

All of these protein complexes contain unique as well as shared proteins (Helmlinger & Tora 2017). Although there is no clear evidence for Mediator subunits to be involved in the known regulatory protein complexes except for the Mediator itself as an entity, the possibility that regulatory complexes containing Mediator with only some Mediator subunits complemented with additional proteins cannot be ruled out.

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 (He & Weintraub 1998). Mediator is disassembled from promoters following the phosphorylation of Pol II carboxy-terminal domain (PolII CTD) by TFIIH (Wong et al. 2014). 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 (Wong et al. 2008). 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 (Connelly et al. 2018; Luc & Tempst 2004).

Protein interactions are regulated by PTMs (Duan & Walther 2015). Protein-protein interactions are frequently functionally connected (Duan & Walther 2015). More than 60 % of PTMs are found in regions that are engaged in specific protein-protein interactions (Lu et al. 2013). Lysine acetylation is one of the most frequent PTMs found in mammals (Lu et al. 2013).

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 bioinformatic searches across stored data (e.g. MegaMotifBase (http://caps.ncbs.res.in/MegaMotifbase/index.html) including 1032 protein structure families and 1194 superfamiles) (Pugalenthi et al. 2008). Besides 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 (Eliezer 2007). These proteins showing signs of intrinsic structural disorder are now in the focus of biological studies.

2.5 *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 (Brenner 1974), 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 (Reinke et al. 2013). It was the first multicellular organism that had its whole genome sequenced (Hodgkin et al. 1995). The community of researchers working on *C. elegans* undertook the pioneering task of open sharing of methodology and data in the scientific community. The discoveries made using the model *C. elegans* further multiplicated the versatility and power of the system for unbiased biological research.

C. elegans has two sexes, hermaphrodites and males, that develop from the embryonic stage until adulthood in approximately four days (dependent on the culture conditions) (Brenner 1974). The developmental scheme of all somatic cells is invariant (with small well defined exceptions) and a large number of regulatory proteins necessary for the proper development of most cells is known (Salipante & Horwitz 2007). This was made possible by detailed analyses of thousands of genetically characterized mutants, and after the discovery of RNA interference by large scale as well as focused RNAi screens that attributed characterized developmental and metabolic functions to the majority of all confirmed as well as informatically predicted protein coding genes.

The complete knowledge of the genome including its intergenic and intronic regions and the comparison to similar data gathered by analyses of related as well as more distant nematode species (*C. brenneri*, *C. reamanei*, *Pristionchus pacificus*) allowed powerful informatic predictions of conserved regulatory sequences.

The *C. elegans* genome is, in comparison to mammalian genomes very condensed. However, approximately 40% of *C. elegans* genes are orthologous of mammalian genes. Despite that the gene number is close to 20 000 in both nematodes and mammals, the nematode genome contains shorter genes mainly due to the presence of very short exons as well as introns and intergenic sequences. Approximately 20% of *C. elegans* genes are organized in operons. In this case the expression of the entire operon is regulated by the promoter positioned in front of the first gene in the operon. All genes of the operon, including the first gene, are transspliced with splicing leader sequence, that is encoded separately in genomic arrays. The splice leader sequences differ for the first gene in operon, which is transspliced with SL1 sequence and genes that are second or other downstream genes in the operon are transspliced with SL2 sequence. Since the splice leaders remain attached to mRNAs, it is easily possible to predict from the attached splice leader, if the gene is encoded separately or is organized in an operon. In some operons, genes organized within the operon retain their own promoter and may be expressed under the regulation of the promoter of the first gene in the operon as well as under the regulation of their own internal promoter. Messenger RNAs expressed from such genes are found in this case with both SL1 and SL2 (Blumenthal 1995; Evans et al. 1997; Blumenthal 2005). Operons including genes with internal promoters are referred to as hybrid operons.

The large scale analyses allowed minute time scale resolution of the transcriptome (Reinke et al. 2013). For analysis of transcription regulation, *C. elegans* contributed enormously with detailed developmental studies (e.g. on HLH gene) and recently by

employing large scale methods for visualization of regulatory sequences capable of activating gene expression (promoterome), binding sites for individual TFs, regions occupied by activated Pol II, and regions with specific chromatin modifications (H3K4me3, H3K27ac, H3K36me3, H3K27me3, H3K9me3) (Reinke et al. 2013).

The data are freely accessible through several *C. elegans* projects such as modENCODE (Gerstein et al. 2010; Allen et al. 2011; Roy et al. 2014) and databases like WormBase and include the analyses of 5' and 3' UTRs, mapping of used splice leaders, microRNAs expression and targets.

Regulatory potential of proteins can be very effectively studied in the *C. elegans* models system which allows to study protein functions in regulation of development on the level of the entire organism whose all cells can be viewed using microscopy and the organism can be easily studied during its entire development and life span.

This makes *C. elegans* model system very appealing for functional studies on regulation of gene expression important for cell differentiation and development of the entire organism.

2.6 Contemporary challenges in understanding Mediator complex functions and its regulatory potential

The concept of the Mediator complex arose from studies focused on the identification of TCoFs critical for the regulatory potential of distinct TFs in yeast and in mammals. This led to the identification of TF-specific coactivator proteins and protein complexes that differed in their composition but included overlapping proteins. It was soon realized that these protein complexes were formed by identical or orthologous proteins. Some complexes can also be deficient for some subunits like Mediator complexes lacking the kinase module, which

were the first that were proven to exist (Malik & Roeder 2000). Bioinformatic tools allowed unification of individual proteins constituting the Mediator complex associated with different TFs over the metazoan phyla (Bourbon et al. 2004; Bourbon 2008) and led to a unified nomenclature of Mediator subunits (Rachez & Freedman 2001; Bourbon et al. 2004).

However, there are many remaining questions and challenges in understanding the precise role of the Mediator complex and its individual subunits in the mechanism of transcriptional regulation. Is Mediator critically needed for all types of gene expression? Are all known subunits necessary for Mediator functions? Are there additional still unknown Mediator subunits? Is Mediator indispensable for basal transcription? Are there specialized forms of Mediator complexes necessary for some types of gene expression related to the regulation of development? These questions may be addressed in the model system *C. elegans* which enables versatile experimental strategies to be done and allows the analysis of specifically designed lines to be analyzed *in toto*, on the level of the entire organism during its entire development and entire life span.

From structural and proteomic studies, it is known that the Mediator complex possesses a high degree of structural flexibility and a variable subunit composition. This variability may be due to the steric effects of individual subunits as well as their presence or absence (Poss et al. 2013).

Studies made on yeast identified subunits important for a wide range of gene expression. They included Med17 and Med21 (Thompson & Young 1995; Holstege et al. 1998). Some yeast Mediator subunits are likely to be non-essential since their single mutants were viable at laboratory conditions. The set of likely unessential subunits for yeast viability at special conditions is surprisingly broad and includes Med1, Med2, Med3, Med5, Med9, Med15, Med16, Med18, Med19, Med20, Med31, and all subunits composing CDK module: Med12 (srb8), Med13 (srb9), CDK8 (srb10), and CycC (srb11) (Dettmann et al. 2010). In

budding and fission yeast as well as in human HeLa cells, mass spectrometry analyses indicate that most Mediator subunits are present in stoichiometric quantities but some subunits may be over- or under-represented (Kulak et al. 2014). Keeping with this, mammalian Mediator complexes with incomplete set of subunits were reported. The subunits that were missing in incomplete Mediator complexes include Med1(TRAP220) (Malik et al. 2004) and Med1 and Med26 (Taatjes & Tjian 2004; Allen & Taatjes 2015). Mediator complexes isolated form HeLa cells and cells with features of stem cell-like character contained full complement of Mediator subunits (Poss et al. 2013). In striking contrast to this, paired samples of undifferentiated cell cultures and cell cultures with induced differentiation showed decreased representation of several Mediator subunits. This was observed in the case of MED14, MED18, MED12, cdk8, MED26 in myotubes (compared to original culture of myoblasts) (Deato et al. 2008) and MED1, MED6, MED7, MED12, MED14, MED16, MED18, MED23, and CDK18 in in hepatocytes compared to original hepatoblast cell culture (D'Alessio et al. 2011). In HeLa cells, quantitative mass spectrometry analyses of Mediator complexes isolated by immunoprecipitation using Med10, Med26, Med28, Med29 tagged with FLAG sequence used for pull-down of studied proteins together with their protein interactants isolated Mediator subunits in similar or equimolar ratios with only small exceptions. The proteins that showed irregular representation included MED30, which was found in doubled ratios when pull-down by Med28. Two Mediator subunits, Med26 and Med29 were recovered more efficiently in their own pull-down experiments. On the other hand, Med31 was immunoprecipitated more efficiently in complexes with Med10 and Med25 but were immunoprecipitated in lower quantities in the remaining pull-downs (Paoletti et al. 2006). From these experiments, it seems likely that Mediator complexes with specialized functions do exist in differentiated cells and in contrary, the cells with a stem-like character

may be critically dependent on the presence of complete Mediator complex. This is opening Mediator complex as a feasible target for molecular interventions for cancer therapy.

In contrary, MED14 which forms a structural frame of Mediator complex is critical for both basal and activated transcription (Cevher et al. 2014).

The ternary structure of Mediator complexes bound to five different TFs (SREB Mediator, VP16-Mediator, TR-Mediator, VDR-Mediator, p53-Mediator) and Mediator complex not associated with a TF showed distinct sterical conformations (Poss et al. 2013).

It can be expected that this leads to the formation of a multipotent molecular structure for very diverse protein-protein interactions. The variability of this molecular interplay may well include association of proteins primarily not associated with the regulation of gene expression that was proposed based on binding studies by Kostrouch and colleagues and referred to as the free protein proteome regulation of gene expression (Kostrouch et al. 2014). This is possible because a fundamental feature of the Mediator subunits is the presence of IDRs that are to a certain degree positionally conserved between species, while others evolved in a phylum or species specific way (Nagulapalli et al. 2016). Nevertheless, the structurally firm protein-protein interactions are likely to be involved as well, provided that the primarily cytoplasmic protein happens to be present inside nuclei.

Other physical interactions are also likely to exist. In yeast, Med3 and Med15 form amyloid-like protein aggregates under H_2O_2 stress conditions. The amyloid formation can be induced by overexpression of Med3 or the glutamine rich domain of Med15. This subsequently leads to the loss of the Med15 module from Mediator and induces a change in the stress response (Zhu et al. 2015).

The Mediator complexes contact a wide range of TFs using a fuzzy protein interface (Brzovic et al. 2011; Warfield et al. 2014). It can be therefore anticipated that additional

proteins with a similar protein-protein interaction potential have the capability to interact with Mediator subunits if they are translocated into the nucleus.

In *C. elegans*, numerous individual Mediator subunits were shown to be associated with specific functions (reviewed in (Grants et al. 2015)). *C. elegans* in this respect offers a powerful system that enables the study of individual proteins involved in developmental regulation which reflects gene expression states.

MED28 has a special position in-between Mediator subunit proteins for its proven dual regulatory role, as a Mediator subunit (Sato et al. 2004; Beyer et al. 2007) and in the cytoplasm, on the level of the cytoskeleton (Gonzalez-Agosti et al. 1996; Wiederhold et al. 2004; Lee et al. 2006; Lu et al. 2006; Huang et al. 2012a).

It can be anticipated that proteins primarily interacting with MED28, if translocated into the nucleus, may be able to bring cytoplasmic regulatory interactions towards the regulation of gene expression. Recent reports identified numerous proteins forming the Mediator as targets of mutations and alterations in human cancers (Shaikhibrahim et al. 2014; Klumper et al. 2015; Bragelmann et al. 2016; Syring et al. 2016; Offermann et al. 2017). A protein that is likely to be functionally connected with MED28 is MED30. MED30 was recently identified as an upregulated gene in stomach cancer connected with cancer proliferative properties (Lee et al. 2015) and development of cardiomyopathy in mice carrying a missense mutation in the first exon (Krebs et al. 2011). MED28 was also connected with cancer behavior and migration of cancer cells (Huang et al. 2012a).

The work constituting this thesis focused at the elucidation of the orthologue of MED28 in *C. elegans*. To do this, the protein believed to be the nematode MED28 orthologue (W01A8.1) was shown to be in fact the orthologue of mammalian perilipins (Chughtai et al. 2015). Subsequently, the real orthologue of MED28 (F28F8.5) was identified (Kostrouchova et al. 2017) and opened for further analysis.

The phenotypes listed in WormBase after RNAi downregulation of *F28F8.5* are similar to those of *mdt-30*, namely a dumpy phenotype (dpy), burst through vulva phenotype and a locomotion defect but not a germline defect (Wormbase WS 263, accessed on March 29, 2018). *mdt-30* is organized in an operon together with F44B9.8 which is an orthologue of human RFC5 (replication factor C subunit 5) and its inhibition by RNAi leads to embryonic defects. Similarly as *mdt-28*, *mdt-30* is likely to be expressed independently from the operon since it is trans-spliced with both SL1 and SL2 splice leaders (Wormbase WS 263, accessed on March 29, 2018).

The identification of the nematode MED28 orthologue opens nematode MED28 for further studies using the powerful techniques of *C. elegans* model system. This may contribute to better understanding of the regulatory potential of Mediator subunit 28 in regulation of gene expression and development.

3. THESIS HYPOTHESIS, GOALS AND THESIS FOCUS

The goals of this thesis were to study an evolutionarily conserved protein whose function would be both nuclear and cytoplasmic and to find out whether it would be regulatable by epigenetic modification. Bioinformatic analyses indicated a possible misclassification of the 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.
- Using valproic acid as an acetylation tool to test its effect on the already proven *C. elegans* MED28 orthologue.

In the experimental work that forms the basis of this thesis, the hypothetical predicted protein originally proposed to be the *C. elegans* orthologue of MED28 (Bourbon et al. 2004) (named in accordance with *C. elegans* gene nomenclature MDT-28) was studied and shown to

be in fact the sole orthologue of mammalian perilipins, that was believed to be absent in nematode genomes (Chughtai et al. 2015). Next, the likely *C. elegans* orthologue of MED28 (F28F8.5) was identified and consequently confirmed experimentally. It was shown that F28F8.5 is the real orthologue of MED28 (and renamed with the approval from WormBase as MDT-28). It was shown that MDT-28 has conserved dual cytoplasmic as well as nuclear localization indicating that the potential of MED28 orthologues to connect cytoplasmic events with the regulation of gene expression is conserved between mammals and nematodes (Kostrouchova et al. 2017). It was shown that *C. elegans* MDT-28 is an acetylated protein and the level of its acetylation is sensitive to treatment with the inhibitor of histone deacetylases, valproic acid.

4. METHODS

4.1 Sequence analysis

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 (Chughtai et al. 2015; Kostrouchova et al. 2017; Kostrouchova et al. 2018).

4.2 Strains, transgenic lines and genome editing

C. elegans strains and transgenic lines

The wild type Bristol N2 strain was used wherever not otherwise specified or one of the following transgenic lines:

1. *C. elegans* transgenic lines made using the 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 (Chughtai et al. 2015).

1b. *C. elegans* transgenic line KV3 (8418) propagated as a heterozygous line; one allele codes for $\boldsymbol{P_{F28F8.5}}$ ***(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 (Kostrouchova et al. 2017). 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 ***$P_{F28F8.5}$(V:15573749)::gfp::F28F8.5*** (this allele has edited *F28F8.5* with *gfp* tagged to the N-terminus of the encoded protein in the position *V:15573749*, allele is named "edited *gfp*::*F28F8.5*") (Kostrouchova et al. 2017).

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** (Chughtai et al. 2015).

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 (Kostrouchova et al. 2017).

2c. *C. elegans* transgenic line expressing GFP alone under *nhr-180* promoter. This line was used for control of GFP::MDT28 pull-down experiments (Kostrouchova et al. 2018).

Genome editing

The 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, was performed using the CRISPR/Cas9 system as developed and described by (Dickinson et al. 2013; Dickinson et al.

2015; Ward 2015; Dickinson & Goldstein 2016) with modifications (Chughtai et al. 2015; Kostrouchova et al. 2017).

Genome editing of *F28F8.5*

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 (the rescue repair template plasmid), which was prepared using the modified vector pDD28 (Addgene, Cambridge, MA, USA). This modified plasmid contains 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.

A second plasmid containing sgRNA was prepared using the modified pJW1219 plasmid (Addgene, Cambridge, MA, USA) and then co-injected together with the first plasmid. The second plasmid carries the CRISPR/Cas9 system that allows the successful insertion of the gene modifying construct to the target genomic location guided by the single-stranded guide RNA (sgRNA).

Three additional markers, genes coding for mCherry under three cell specific promoters that allow visualization of successful transfer of the CRISPR/Cas9 carrying plasmid were also co-injected.

This strategy was designed to allow successful insertion of the construct to the intended genomic location visualized by the expression of GFP from the construct. Since the *gfp* gene in the construct is promoterless, GFP cannot be expressed from extrachromosomal arrays that are formed in the first transgenic generations.

As a result, positive markers (Rol phenotype and mCherry fluorescence) indicate successful transfer of transgenes, and weak GFP fluorescence in the cytoplasm indicates the successful insertion to the target genomic location (now gaining the *F28F8.5* promoter for GFP expression). This step was designed to create animals lacking the expression of F28F8.5 since the endogenous *F28F8.5* is separated from its natural promoters (both based on the operon and the internal promoter of its hybrid operon) and leads to animals with null expression of F28F8.5.

The second step included activation of the Cre/LoxP system for excision of SEC (on both alleles) by heat shock induced expression of Cre recombinase. Integrated nematodes were selected after 3 days based on the absence of the Rol phenotype, the absence of the expression of mCherry markers and the ability to survive on plates containing hygromycin. The detailed description of the technique is described in supplementary information connected to the primary publication by (Kostrouchova et al. 2017).

Genome editing of *W01A8.1*

A similar strategy was used for editing of *W01A8.1* to the form W01A8.1::GFP (GFP is at the C-terminus of the protein) and for 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 be formed in the natural or close to natural ratios. This allowed the preparation of the following lines: W01A8.1a/c::GFP and W01A8.1b::GFP. *W01A8.1* isoforms a and c have identical 3' ends which both can be expressed from *W01A8.1a/c::gfp*. In all experiments, the modified synonymous codons of *W01A8.1* were used in the sequence in order to block CRISPR/Cas 9 based modification of modified regions back to the wild type forms. The detailed description

of the technique is provided in supplementary information connected to the primary publication (Chughtai et al. 2015).

4.3 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 of *C. elegans* and standard biochemical methods and included: RNAi, Fecundity and brood size assays, PCR including quantitative PCR, microinjections for preparation of transgenic lines and for downregulation 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.

General methods 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.

Methods of RNA interference

Methods of downregulation of gene expression of genes in focus were derived from methods worked out by Dr. Andrew Fire and collaborators (Tabara et al. 1999; Timmons et al. 2001) with modifications (Vohanka et al. 2010). For microinjection RNAi protocol, the double stranded RNA (dsRNA) was prepared by two separate *in vitro* transcription reactions directed at the opposing ends of the selected silencing sequence whose products were mixed together, denatured by heating to 75 °C for 10 min and allowed to anneal by slowly cooling to

room temperature during approximately 30 min. The resulting dsRNA was assayed for its quality by agarose gel electrophoresis, its concentration was estimated from gels as well as using a UV spectrophotometer and the solution with a concentration of approximately 1 μg/μl was used for microinjections.

The feeding RNAi protocol was based on feeding nematodes were fed with bacteria producing dsRNA of the target gene under induction by isopropyl β-D-1-thiogalactopyranoside (IPTG). A special bacterial strain with active T7 polymerase induced by IPTG and transformation with a vector with attached T7 recognition sites on both ends of the subcloned target sequences were used. Selection of bacteria carrying the desired vectors was done by Ampicilin and Ampicilin resistance gene in the RNAi vector. The cultures were grown to OD600 ≈ 1.0; 900 μl of culture was poured onto NGM agar plates to completely cover the surface and 750 μl of the suspension was removed to leave 150 μl of the suspension on the plates. The bacteria were allowed to grow and were induced overnight at room temperate (∼22◦C). The production of dsRNA was then induced by IPTG and experimental and control animals were transferred on the induced plates and cultured for the given exposure time.

Transcript quantification

Quantitative polymerase chain reaction (qPCR) was performed with cDNA prepared from total RNA using the Roche Universal Probe Library technique (Hoffmann-La Roche, Basel, Switzerland). The levels of the expression of assayed genes were normalized against *ama-1*.

Single animal polymerase chain reaction

Single animal PCR was used for verification of all transgenic lines. Selected animals with were removed from microscopic slides after their phenotypical characterization and transferred into caps of PCR tubes with 4 µl of solution of Proteinase K (20 mg/ml) diluted 1:333 in Barstead Buffer (resulting in Barstead Lysis Buffer which consists of 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% (v/v) NP40 (Nonidet P-40), 0.45% (v/v) Tween-20, 0.01% (w/v)) supplemented with Proteinase K at the final working concentration of 200 µg/ml. The tube was closed and the resulting sample was retrieved by a pulse centrifugation. After a short freezing step (10 min. at -70º C), the tube was heated for 1 hour at 65º C and additional 15 min. at 95º C. The analyzed genomic regions were amplified by PCR and analyzed using agarose gel electrophoresis for their length. The genomic regions were determined by DNA sequencing.

Microinjections

Microinjections of plasmids, DNA amplicons or dsRNA into gonads of young adult hermaphrodites were done using an Olympus IX70 microscope equipped with a Narishige microinjection system (Olympus, Tokyo, Japan) as described (Tabara et al. 1999; Timmons et al. 2001; Vohanka et al. 2010).

LipidTox staining

The staining by LipidTox was done according to the method described by O'Rourke and colleagues (O'Rourke et al. 2009) with small modifications. Approximately 200–500 animals were separated from residual bacterial food by several washes with deionized water and following centrifugations at 1,500 ×g. The samples were then treated with 500 µl of 2x MRWB (160mMKCl, 40mMNaCl, 14mMNa2EGTA, 1mM Spermidine 3HCl, 0.4mM Spermine, 30 mM NaPIPES pH 7.4, 0.2% beta-mercapto ethanol) and 100 µl of freshly

prepared 20% (w/v) formaldehyde. The final volume was adjusted up to 1 ml with 1x PBS. After 60 min. fixation, animals were pelleted at 1,500×g and washed 3 times with 1 ml Tris–HCl buffer (100 mM, pH 7.4). The sample volume was reduced to 100 µl and supplemented with 650 µl o f Tris-HCL buffer and 250 µl of reduction buffer (100mMTris-Cl pH 7.4, 40mMDTT). Following 30 min. incubation, the samples were washed 3 times in 1x PBS and the volume adjusted to 500 µl and mixed with 500 µl of LipidTox (Red) diluted 1:500 dilution), incubated for 60 min. and used for analysis by fluorescent microscopy.

Valproic acid treatment

Synchronized *C. elegans* L1 larvae were transferred on Nematode growth medium (NGM) plates containing 0, 1 or 5mM of valproate (Valproic acid sodium salt, Sigma-Aldrich), a suspension of OP50 bacteria (containing the corresponding concentration of valproate) and incubated for 4h at room temperature (RT, 22°C). The samples were then either used for microscopic analyses, determination of proteins by Western blot analyses, or for immunoprecipitation using the GFP-Trap method (ChromoTek Inc., Hauppauge, NY, USA, https://www.chromotek.com).

4.4 Microscopy

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

Samples were analyzed by 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.

For FLIM acquisitions, the single photon counting signal from the internal hybrid detectors operated at confocal mode was simultaneously processed by HydraHarp400 TCSPC electronics (PicoQuant, Berlin, Germany). The information about the arrival times of pulse-excited photons was transformed into TTTR data format, a program developed by Dr. Aleš Benda and made available to all users via the website of PicoQuant, Berlin, Germany (https://www.picoquant.com/images/uploads/page/files/14528/technote_tttr.pdf). TTTR data analysis allows proper handling of the bidirectional harmonic scan with line accumulation and/or sequential excitation. The data structure, program description and user instructions are also freely accessible at https://github.com/PicoQuant/PicoQuant-Time-Tagged-File-Format-Demos/blob/master/PTU/Matlab/Read_PTU.m).

For data processing, the signals from both time synchronized channels were combined and presented using a false color scale as the average photon arrival time. This strategy allows separation of the fluorescent signal of GFP from that of autofluorescence; GFP fluorescence has a lifetime of around 2.4 ns while autofluorescence has the lifetime maxima between 1 and 1.5 ns. The acquired fluorescence signals were presented as false-color images of recorded fluorescence with the lifetime between 1 and 3 ns. The emission signal upon 488 nm excitation was spectrally split into two hybrid detectors with similar intensity at each of them to lower the detector saturation at bright pixels.

Coherent anti-Stokes Raman scattering microscopy

CARS microscopy 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 (Chughtai et al. 2015).

4.5 Image analyses

Image analyses were done using the ImageJ computer program available from ImageJ website with appropriate plug-ins (http://imagej.nih.gov/ij/).

For densitometric analysis of FLIM images of *C. elegans* exposed to valproic acid, the files of control and experimental animals were assembled and analyzed structures were selected and measured using identical settings. The GFP signal amplitude in each pixel was estimated from the average arrival time of photons in that pixel, assuming a two-component model consisting of GFP and the autofluorescence signal. The amplitude of the GFP signal was retrieved from the red channel of the false color images.

4.6 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. Based on bioinformatic analysis we selected several subunits from the head module of Mediator including MDT-6, 11, 27, 29 and 30. All these proteins were prepared as *in vitro* translated radioactively labeled proteins and as proteins expressed in bacteria similarly as described (Kostrouch et al. 2014). The cDNAs of assayed Mediator subunits were prepared from mixed stages *C. elegans* cultures and cloned into the pTNT vector ((Promega, Madison, WI, USA) and expressed in the rabbit reticulocyte TNT-system (Promega, Madison, WI, USA). The i*n vitro* transcribed proteins were labeled using ^{35}S Methionine (Institute of Isotopes, Budapest,

Hungary). Binding studies were done as described in (Kostrouch et al. 2014) with modifications for particular mediator subunits. The method details are described in the attached publication (Kostrouchova et al. 2017).

For bacterial expression, the cDNAs of studied proteins were cloned into the pGEX-2T vector ((Amersham Pharmacia Biotech, Amsterdam, UK), transformed into BL21 *E. coli* cells and the production of proteins were induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA). The FLAG sequence was used for detection by anti-FLAG antibody on Western blots. In the case of MDT-30, the lysate from bacteria producing His_6-MDT-30-FLAG was used directly or purified on HiTrap Chelating HP column (GE Healthcare, Chicago, IL, USA). Proteins produced by the TNT system or bacterial lysates of bacteria transformed with FLAG labeled Mediator subunits were incubated with glutathione-agarose (Sigma-Aldrich, St. Louis, MO, USA) adsorbed with equal amounts of GST or GST-F28F8.5. Bound radioactively labeled proteins were detected using TRI-CARB 1600TR, Liquid Scintillation Analyzer (Packard, Meriden, CT, USA) and by polyacrylamide gel electrophoresis using autoradiography and subsequently by the scintillation counter. FLAG-labeled MDT-30 was determined by Western blot using an anti-FLAG antibody (monoclonal anti-FLAG, M2 (Sigma-Aldrich)) and quantified densitometrically by the ImageJ computer program (https://imagej.nih.gov/ij/download.html) (Schneider et al. 2012).

4.7 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). Equal volumes of experimental samples were incubated with the same amount of magnetic agarose beads

with covalently bound monoclonal single chain anti-GFP antibody and separated from non-bound proteins using a magnetic field, stored as aliquots at 4 °C before being used for further analyses. The amount of bound protein was determined using Western blots with mouse GFP antibody B-2 (at a dilution of 1:222) (sc-9996, Santa Cruz Biotechnology, Santa Cruz, CA) and the level of lysine acetylation was determined by a mouse acetyl-lysine monoclonal antibody 1C6 (at a dilution of 1:1000) (Abcam, Cambridge, MA) and goat anti-mouse secondary antibody (H+L)-HRP conjugate (1 : 10,000) (Bio-Rad).

5. RESULTS

5.1 The hypothetical protein F28F8.5 is the nematode orthologue of Mediator complex subunit 28 and is a critical regulator of *C. elegans* development

(**Kostrouchová M**, Kostrouch D, Chughtai AA, Kaššák F, Novotný JP, Kostrouchová V, Benda A, Krause MW, Saudek V, Kostrouchová M, Kostrouch Z. The nematode homologue of Mediator complex subunit 28, F28F8.5, is a critical regulator of *C. elegans* development. PeerJ 2017, 5:e3390 https://doi.org/10.7717/peerj.3390; IF = 2.118)

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 to the conclusion that F28F8.5 is more likely the true 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 (Figure 3). 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 proteins translated from F28F8.5 isoforms a and b thus have a similar size as mammalian MED28 orthologues. The two isoforms differ only in a two amino acid insertion in the second exon of isoform b. 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 (represented by red horizontal lines in Figure 3).

Figure 3. A multiple sequence alignment of the central conserved part of selected metazoan homologues of MED28 compared with F28F8.5 and aligned with PROMALS (http://prodata.swmed.edu/promals/promals.php). Amino acid residue types are colored according to the Clustal scheme in Jalview. Horizontal red bars indicate predicted alpha-helices. Aligned sequences are: *Caenorhabditis elegans*, O18692; *Trichinella spiralis*, E5RZQ1; *Wuchereria bancrofti*, EJW84794.1; *Pristionchus pacificus*, translated contig of CN657719.1 FG102945.1 CN657262.1 CN656622.1; *Ancylostoma ceylanicum*, A0A016SKV7; *Globodera pallida*, translated CV578368.1;

Panagrolaimus davidi, translated JZ658977.1; *Ixodes scapularis*, B7PAW5; *Drosophila melanogaster*, MED28_DROME; *Homo sapiens*, MED28_HUMAN.

F28F8.5 gene organization

According to WormBase (WS270), *F28F8.5* is organized in a hybrid operon. It can be expressed under the regulation of the promoter of the first gene in the operon or under the regulation of its internal promoter that is localized immediately before *F28F8.5* coding sequence. The operon CEOP5444 contains 4 genes, *F28F8.7*, *F28F8.9*, *F28F8.6* (*atx-3*) and lastly *F28F8.5*.

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.

The strategy exploiting gene editing by the CRISPR/Cas9 system leads to the expression of F28F8.5 tagged by GFP on its N-terminus. Control animals expressing large amounts of GFP from extrachromosomal arrays under the regulation of a strong ubiquitously active promoter of *nhr-180* indicated that the expression of untagged GFP is not harmful for normal development of *C. elegans*. The used strategy yielded heterozygous animals with one wild type *F28F8.5* allele and one allele with disrupted *F28F8.5* which had an inserted gene coding for GFP (*gfp*) after the internal promoter of *F28F8.5* in front of the first codon. This induced the expression of GFP alone but disrupted *F28F8.5* gene separating its coding region from its natural promoters making it promoter-less and by stop codons positioned at the 3’

end of the inserted *gfp* thus forming a null allele of *F28F8.5*. The heterozygous animals and animals with the disrupted *F28F8.5* gene were analyzed separately (see below).

Animals with both *F28F8.5* alleles edited in the form $P_{F28F8.5}$*(V:15573749)::gfp::F28F8.5* (line KV4, 8419) were formed by excision of SEC induced by heat-shock. The line was used for the analysis of F28F8.5 expression at normal or close to normal expressional rates (due to the presence of GFP) since the *C. elegans* lines with both edited alleles were viable and produced viable embryos. The GFP::F28F8.5 pattern was observed ubiquitously in most if not all cells and detected in nuclei as well as in the cytoplasm. The nuclear localization of GFP::F28F8.5 was observed from early embryonic stages to adults in cells that included enterocytes, epidermal cells, pharyngeal, uterine and vulval muscle cells (Figure 4 and 5). In agreement with active expression of endogenous edited genes in the gonad, GFP::F28F8.5 was found expressed in the germline and oocytes (Figure 4 and 5).

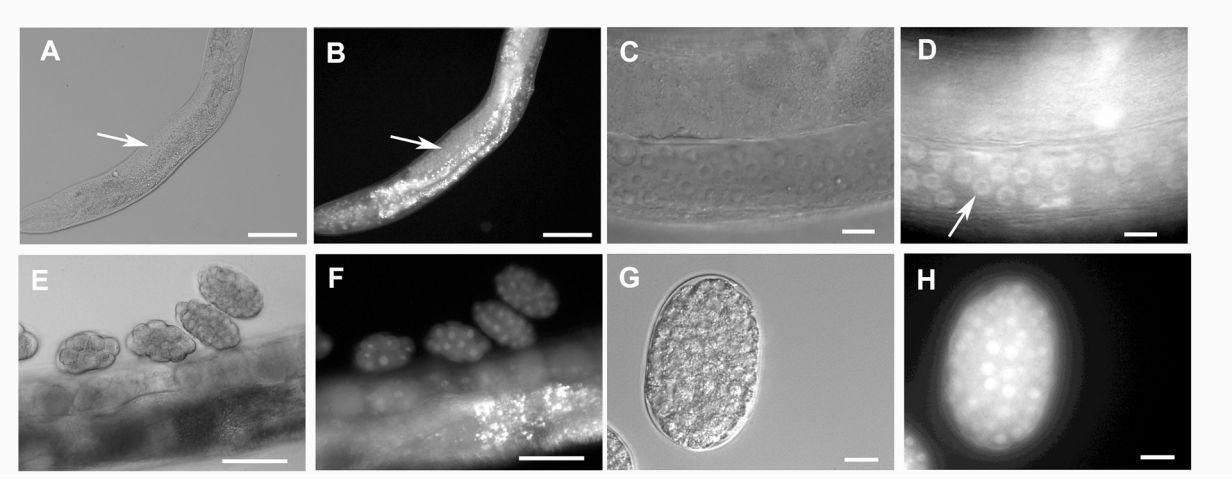

Figure 4. Expression pattern of GFP::F28F8.5 in homozygous animals with edited *F28F8.5* gene. Fluorescent microscopy reveals the presence of GFP tagged to F28F8.5 (at its N-terminus using CRISPR/Cas9 technology) in the mitotic nuclei of the gonad (Panels B and D, indicated by arrows). The expression then continues in nuclei of embryos from the early (Panel F) as well as later embryonic

stages (Panel H). Panels A, C, E and G show parallel images of panels B, D, F and H, respectively. Bars represent 50 µm.

Confocal microscopy confirmed the nuclear as well as cytoplasmic subcellular distribution of GFP::F28F8.5. Since the expression of the GFP-tagged protein from the endogenous alleles is faint in comparison with the known strong autofluorescence of gut granules and other subcellular structures in *C. elegans* present in embryos, all larval stages and adults, FLIM was applied . The expectation was that autofluorescence likely produces a signal with short lifetime fluorescence (color-coded as blue or green) while GFP-specific fluorescence produces a signal with longer lifetime fluorescence of around 2.4 ns (color-coded as yellow or red). In keeping with this, FLIM distinguished the autofluorescent signal from structures such as gut granules from GFP-specific fluorescence clearly detected in oocytes and embryos from the one cell stage throughout the embryonic development to L1 larvae and further in all larval stages to adults (Figure 5).

The experimental setting also allowed a clear detection of GFP::F28F8.5 in the cytoplasm of enterocytes, epidermal and muscle cells and in the cytoplasm of male ray cells (Figure 5 panel I).

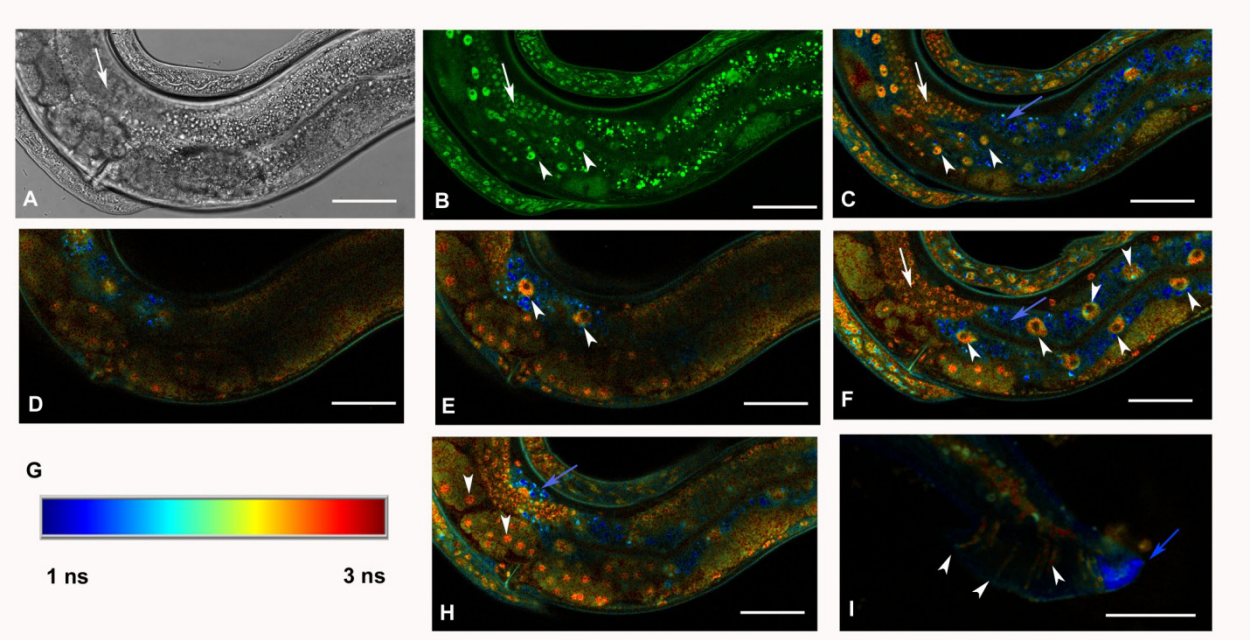

Figure 5. Analysis of GFP::F28F8.5 expression in homozygous animals with edited *F28F8.5* gene by Nomarski optics (Panel A), confocal microscopy (Panel B) and fluorescence lifetime imaging microscopy (FLIM) (Panels C, D, E, F, H, and I). FLIM images are calculated from merged recordings in Channel 1 and Channel 2 (525–585 nm). Panels A to H show images of an adult animal and two L1 larvae with edited F28F8.5. Panels C, D, E, F and H show selected focal planes in FLIM. Panel G shows the calibration table for FLIM in the range of 1–3 ns used in all panels presenting FLIM analysis. Blue areas shown in FLIM pictures represent short lifetime fluorescence presumably corresponding to autofluorescence (blue arrows in panels C, F, H and I). Arrowheads in panels B, C, E, and F indicate nuclei of enterocytes and in panel H nuclei of early embryos with long lifetime fluorescence characteristic for GFP. Panel I shows the distal part of a male expressing GFP:F28F8.5 in male specific structures, in nuclei as well as in rays (marked by arrowheads) indicating that GFP::F28F8.5 is expressed not only in cell nuclei but also in the cytoplasmic structures. Bars represent 50 μm.

The second strategy used for the visualization of the expression of F28F8.5 utilized transgenic lines carrying extrachromosomal arrays consisting of *F28F8.5* internal promoter, *F28F8.5* coding sequence fused in frame on its 3' end with the gene coding for GFP followed by stop codon and artificial 3' UTR. In this case, the transgene led to the expression of F28F8.5::GFP observed in embryos from the twofold stage to adulthood (Figure 6). The cells prominently expressing the transgene included seam cells and enterocytes. The transgene was found localized both in the cytoplasm and nuclei. Generally, the expression of F28F8.5::GFP was stronger that the expression of GFP::F28F8.5 from the edited alleles as expected for genes encoded by extrachromosomal arrays. In some cells, a clear predominance of nuclear expression, especially in the case of enterocytes was observed. The cytoplasmic expression of F28F8.5::GFP was also observed in the excretory cell and its channels. Similarly as the lines with edited *F28F8.5*, the lines with extrachromosomal arrays expressing clearly visible amounts of F28F8.5::GFP were viable and easily propagated indicating that the tagging of F28F8.5 on both N- and C-termini does not critically abrogate F28F8.5 vital functions.

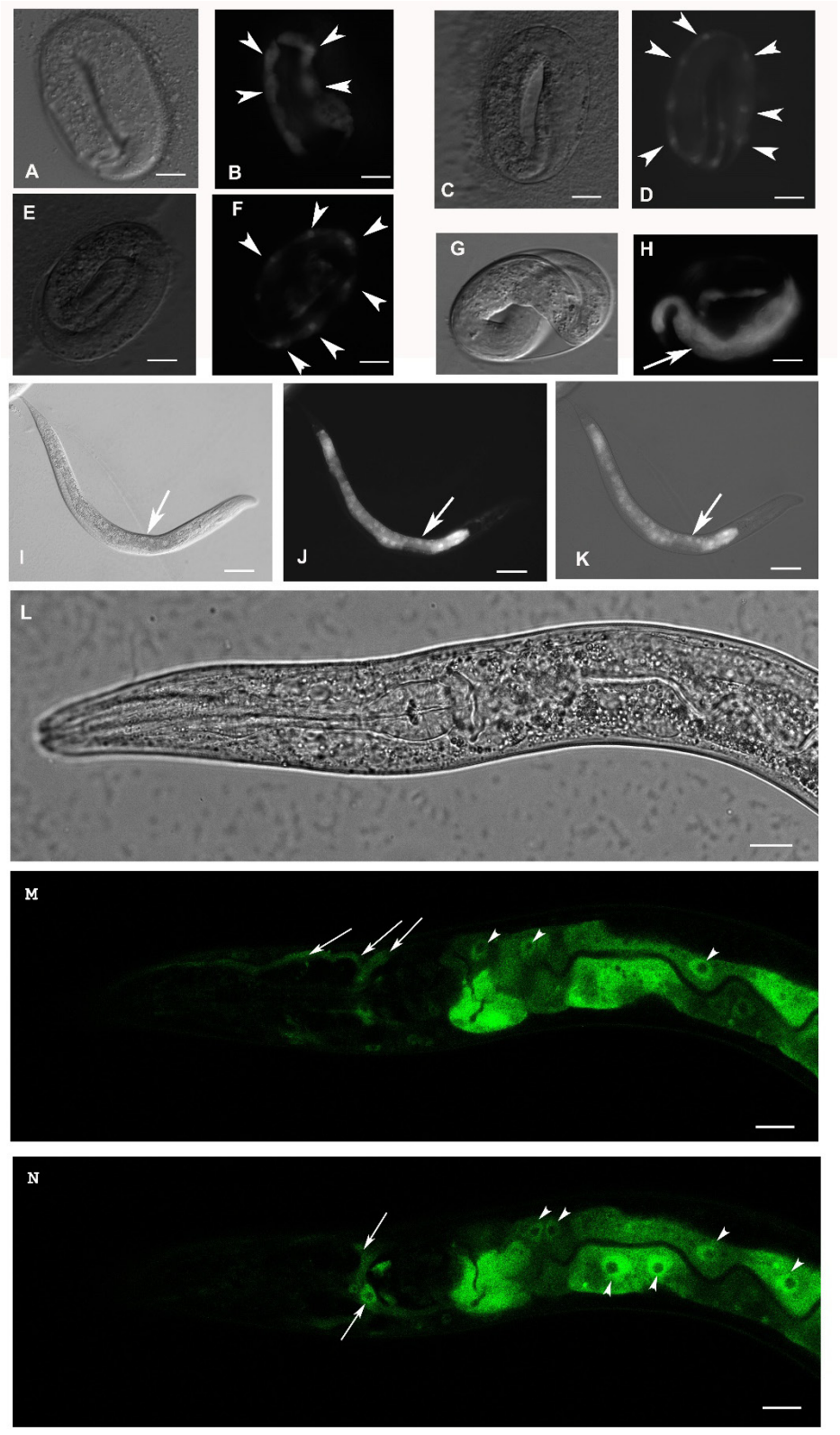

Figure 6. Expression of F28F8.5::GFP from extrachromosomal arrays. Panels A, C, E and D show embryos in Nomarski optics and panels B, D, F and H in fluorescence microscopy. Panels I to K show a L3 larva in wide field microscopy, panel I in Nomarski optics, panel J in fluorescence microscopy and panel K as a composite image of panels I and J. Panels L to N show a L3 larva in confocal

microscopy, panel L in Nomarski optics and panels M and N show two focal planes in confocal fluorescence microscopy in GFP emission recording mode. The picture shows a strong expression of F28F8.5::GFP in nuclei as well as in cytoplasm of enterocytes starting in a two-fold embryo (panel B), Panel D in a two and half-fold embryo and panels f in a three-fold embryo. The GFP fluorescence starts to be recorded in the cytoplasm of two-fold embryo with subsequent re-distribution into nuclei of enterocytes. Panels G and H show the end of embryogenesis when already fully formed L1 larva is shortly before hatching with stronger cytoplasmic expression of the transgene. Panels I to K show strong nuclear and cytoplasmic expression of F28F8.5::GFP in the L3 larva. Confocal fluorescence microscopy (panels M and N) confirms strong cytoplasmic and nuclear localization of F28F8.5::GFP in enterocytes of the L3 larva with accented expression in nuclei of enterocytes (arowheads) and strong expression of the transgene in channels of excretory cell as well as in its cell body including nucleus (arrows). Panels M and N are consecutive focal confocal planes in Z axis. Bars represent 10 μm.

F28F8.5 regulates development

RNAi was used to down-regulate *F28F8.5* expression and to induce possible loss-of-function phenotypes. Analysis of the total progeny of 17 young adult hermaphrodites inhibited for *F28F8.5* function by microinjection of dsRNA into the syncytial gonad lead to visualization of multiple developmental phenotypes in 1127 animals (of total 2567 animals) (44 %). The observed developmental phenotypes included embryonic and larval arrest and a spectrum of less severe phenotypes that included defective molting, protruding vulva that often burst, defective male tail rays (Figure 7), and impaired movement (uncoordinated Unc phenotype).

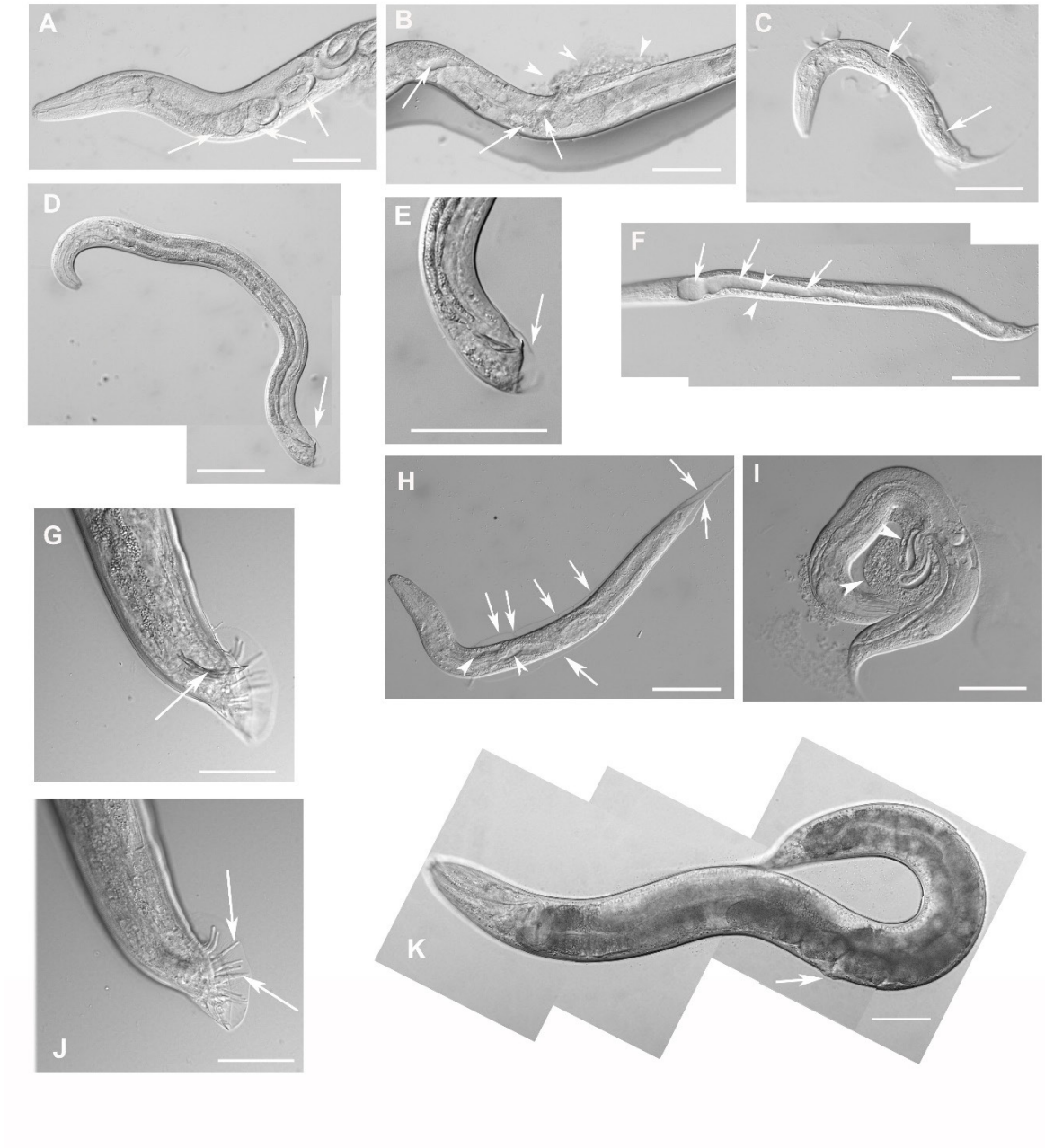

Figure 7. Downregulation of F28F8.5 by RNAi induces developmental defects. Animals developed from parents injected with dsRNA specific for F28F8.5 show retention of normal and malformed embryos (panel A), vacuoles (panels A and B, arrows), herniation and burst through vulva (panels B and I, arrowheads) and defective development of the gonad (panel I). Panel C shows a dumpy phenotype with masses of tissue and vacuoles (arrows) that was common in the progeny of microinjected parents. Panel D shows a male nematode with defects of male specific structures - missing rays and fan and an abnormal distal part of the body (arrow). Panel E shows the magnified distal part of the male nematode in panel D and defective male specific structures (arrow). Panel F shows an atrophic L3 larva with thin enterocytes (arrowheads) and a dilated gut lumen (arrows). Other phenotypes seen included molting defects indicated by arrows in panel H and cellular defects

(indicated by arrowheads). Animals treated by control RNAi were morphologically normal and are shown in panels G, J and K. Panels G and J show the distal part of the body of a male animal with normal appearance of male specific structures. In panel G the arrow marks spicules and in panel J normal sensory rays. Panel K shows a control adult hermaphrodite with normal appearance and fully developed vulva (arrow). All images are in Nomarski optics. Bars represent 50 μm.

The fact that the CRISPR/Cas9 technique in the used setting initially disrupts the *F28F8.5* gene on either one or both alleles enabled to observe the effect of loss of function of F28F8.5 *in vivo* and detect sterility, among other phenotypes, in homozygous mutants created in this way. This indicates that F28F8.5 is 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 (Figure 8). The gonad was irregular, contained masses of undifferentiated tissue and showed problems of directional growth. Defective vulva formation was also observed.

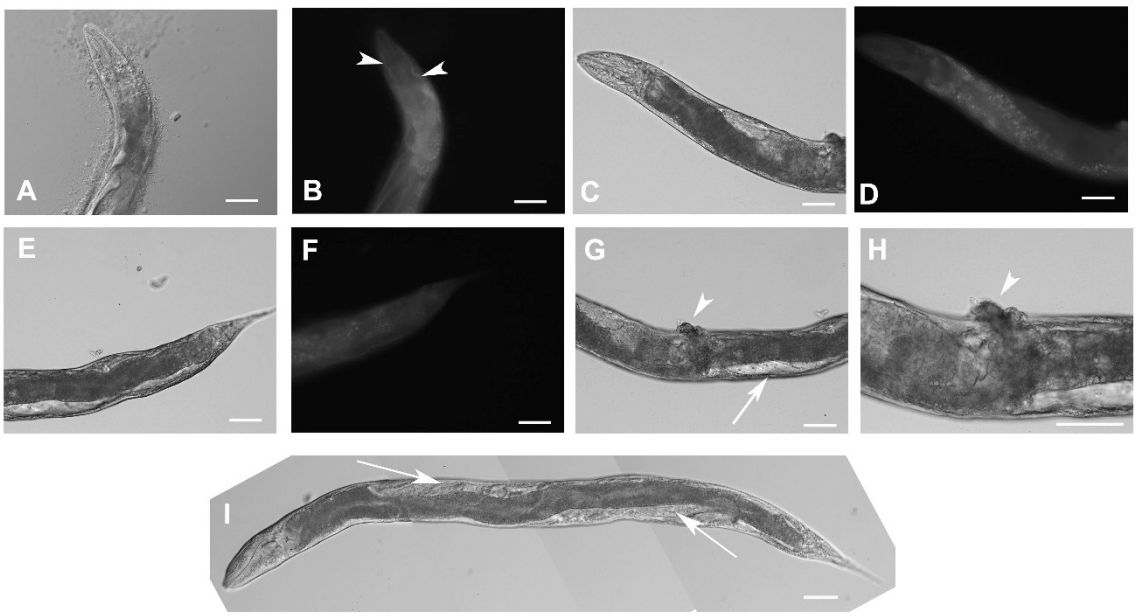

Figure 8. Disruption of F28F8.5 by CRISPR/Cas9 technique. Animals with disrupted F28F8.5 on both alleles express GFP under the regulation of *F28F8.5* promoter. Panels A to H show paired images of animals in Nomarski optics and in GFP fluorescence. Panels A and B show an adult hermaphrodite animal with diffuse fluorescence in cells in the head area including anterior arms of the excretory cell (arrowheads). Panels C, D, E, and F show an adult animal with a malformed gonad, Pvul phenotype, dense gut and diffuse GFP fluorescence throughout the body. Panels G and H show the central part of the body of a hermaphrodite with the Pvul phenotype (arrowhead) and malformation of gonad (arrow). Panel I is composed of three consecutive images showing an adult hermaphrodite animal with severely malformed gonad (arrows), and missing uterus and spermathecae. The fluorescence images show that unlike GFP::F28F8.5, GFP alone localizes diffusely in the cytoplasm and is not found in nuclei. Bars represent 50 μm.

Heterozygous hermaphrodites carrying one edited disrupted allele of *F28F8.5* and one WT allele appeared normal and produced viable embryos (Figure 9). In these animals, the GFP fluorescence originating from produced untagged GFP (expressed under the regulation of *F28F8.5* promoter but separated from the *F28F8.5* coding sequence) was diffusely cytoplasmic. In some cells of produced embryos, there was clear nuclear accumulation of GFP fluorescence indicating occasional spontaneous excision of SEC. Heterozygous animals

produced segregated animals with wild type MDT-28, heterozygous animals and animals with edited disrupted *F28F8.5* on both alleles producing untagged GFP. Occasionally, animals with one edited disrupted *F28F8.5* allele and one edited *F28F8.5* allele (with excised SEC) were also detected. They were recognizable by the combination of the following phenotypic markers: Rol phenotype, expression of GFP in nuclei and lack of developmental phenotypes (Figure 9).

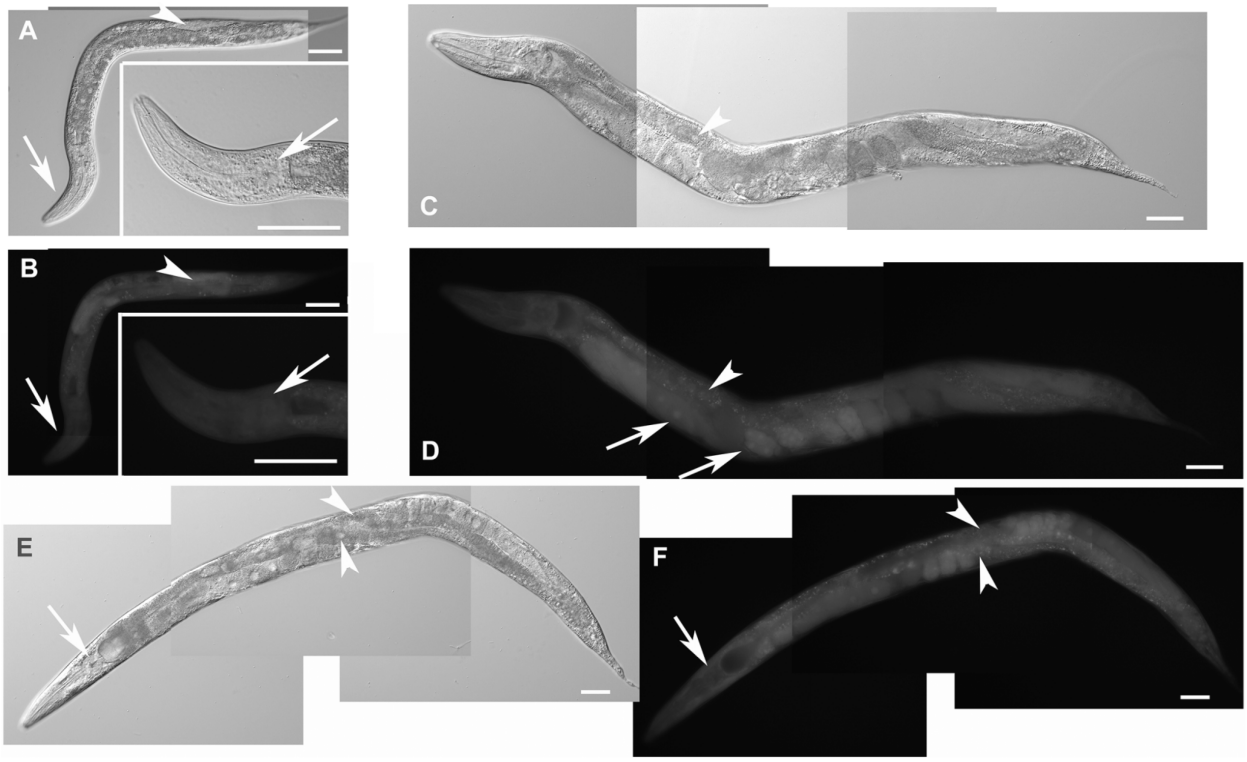

Figure 9. Heterozygous animals with one edited disrupted allele of F28F8.5 and one WT allele.
Heterozygous hermaphrodites carrying one edited allele of F28F8.5 and one WT had grossly normal appearance and could be recognized by Rol phenotype, presence of embryos, weak mostly cytoplasmic GFP fluorescence and absence of nuclear localization of GFP fluorescence. Panels A (Nomarski optics) and B (GFP fluorescence) show an L3 larva with weak fluorescence (panel B, arrowhead points at the gonad and arrows point at the head and pharynx). Inlets show head area at higher magnification (rotated 90 clockwise). Panels C and D show an adult hermaphrodite animal (C in Nomarski optics and D in GFP fluorescence) with weak cytoplasmic fluorescence in most cells. The arrowhead in panel D points at the nucleus of an enterocyte in focal plane that is devoid of GFP fluorescence. Arrows indicate two embryos with GFP fluorescence accumulated in nuclei which is

most likely the result of spontaneous SEC self-excision. Panels E and F show an adult hermaphrodite in Nomarski optics (panel E) and GFP fluorescence (panel F). Arrows indicate the head area with diffuse intracellular fluorescence visible in panel F. Arrowheads point at two nuclei of enterocytes in focal plane that are also devoid of fluorescence. In contrast to the animal shown in panels C and D, the animal shown in the panel E and F contains embryos that have mostly diffuse cytoplasmic expression of GFP. Bars represent 50 mm.

F28F8.5 interacts with Mediator complex subunits

Interaction between F28F8.5 and other Mediator complex subunits was found using GST-labeled F28F8.5 and radioactively labeled MDT-6 or FLAG-tagged MDT-30. This proves that F28F8.5 possesses biding properties expected for a MED28 orthologue

(Figure 10).

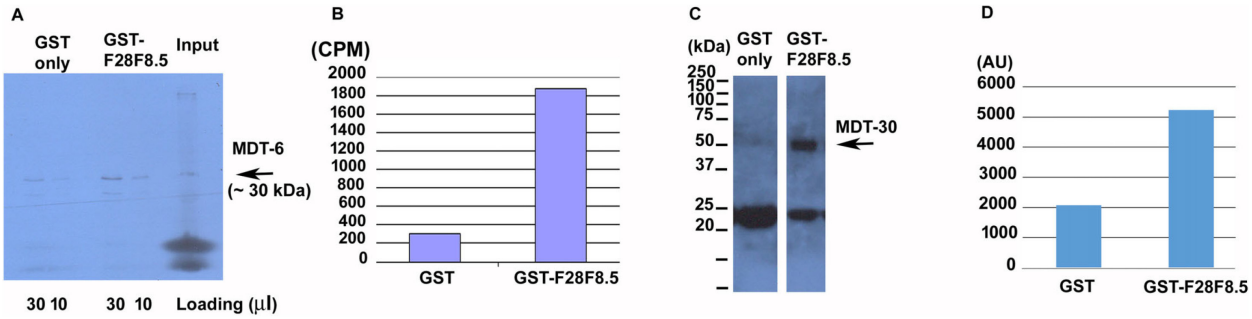

Figure 10. Binding of F28F8.5 to MDT-6 and MDT-30 in vitro. GST-F28F8.5 or GST alone were expressed in bacteria and purified using glutathione–agarose beads that were incubated with 35S-Methionine-MDT-6 produced using rabbit reticulocyte lysate (A and B) or His6-MDT-30-FLAG expressed in bacteria and purified using a nickel column (C and D). Panels A and C show fractions bound to glutathione–agarose beads resolved by polyacrylamide gel electrophoresis and visualized by autoradiography (panel A). For quantification, dried gel areas corresponding to proteins detected by radioactivity were excised and the radioactively labeled MDT-6 was determined using scintillation counter (panel B). Panels C and D show the interaction of FLAG-MDT-30 with GST-F28F8.5 or GST alone. FLAG-MDT-30 pulled down by GST or GST-F28F8.5 was determined by Western blot using an anti-FLAG antibody (panel C) and by densitometry (panel D). Both assayed Mediator subunits, MDT-6 and MDT-30 bind GST-F28F8.5 preferentially in comparison to GST only.

5.2 Valproic acid decreases the nuclear localization of MDT-28 – the intracellular localization of MDT-28 is regulatable

(Kostrouchová M, Kostrouchová V, Yilma P, Benda A, Mandys V, Kostrouchová M. Valproic Acid Decreases the Nuclear Localization of MDT-28, the Nematode Orthologue of MED28; Folia Biol (Praha). 2018;64(1):1-9; IF = 1.044)

To support the concept of a dual regulatory function of MED28 orthologues, it was 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, the amino acid sequences of MED28 orthologues were analyzed as possible targets of lysine acetyltransferases. It was found that the most C-terminal lysines are likely targets of acetylation by CBP (Table 1).

Table 1. Predicted acetylation sites of MED28 orthologues

Species	ID	Position	Peptide	HAT	Score	Cutoff	Stringency
C. elegans	H9G301.1 mdt-28	198	EAHPNAG**K**HFTT***	CREBBP	1.548	1.348	M
T. spiralis	tr|E5RZQ1|E5RZQ1_TRISP	163	KLTCSDW**K**IALES**	CREBBP	1.355	1.348	M
W. bancrofti	EJW84794.1	160	LSDLGAE**K**NSQGFE*	CREBBP	1.851	1.785	H
D. melanogaster	Q9VBQ9.1	189	ISRQMPP**K*******	CREBBP	2.161	1.785	H
H. sapiens	Q9H204.1	176	ANIPAPL**K**PT*****	CREBBP	2.165	1.785	H
M. musculus	Q920D3.2	176	ANIPAPLKQT*****	CREBBP	2.532	1.785	H
T. rubripes	XP_003972471.1	178	NLPPAPL**K**PS*****	CREBBP	2.698	1.785	H
X. tropicalis	AAI55039.1 med28	167	ANIPAPM**K**PT*****	CREBBP	2.165	1.785	H
C. intestinalis	XP_002124297.1	141	GAGSQQM**KK******	CREBBP	3.391	1.785	H
C. intestinalis	XP_002124297.1 28-like	142	AGSQQMK**K*******	CREBBP	3.048	1.785	H
T. adhaerens	XM_002117398.1	105	QQDLTKW**K******	CREBBP	2.992	1.785	H

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

The *C. elegans* line KV4, prepared earlier, expressing GFP::MDT-28 from its 2 alleles was used for the following experiments.

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 (Figure 11).

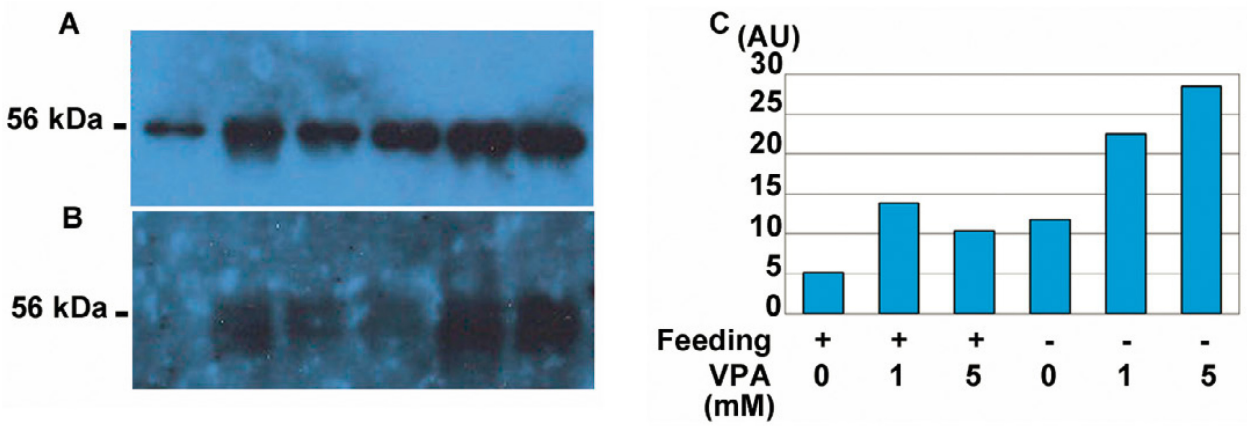

Figure 11. Detection of acetylated lysines of MDT-28 in fed or starved synchronized L1 larvae and incubated for 4 h with 1 mM VPA or 5 mM VPA or without VPA. Panels **A** and **B** show Western blots of immunoprecipitated GFP::MDT-28 pulled-down by the GFP-Trap system and probed with anti-GFP antibody (panel A) and anti-acetylated lysine antibody (panel B). Panel **C** shows the densitometric analysis of the levels of immunodetected lysine-acetylated GFP::MDT-28 shown in panel B normalized for the expression of GFP::MDT-28 with subtracted background staining and adjusted for loading volumes (10 µl in the first three lanes in panel A representing fed cultures and 20 µl in all the remaining lanes). The Western blot and its analysis is one of two independent experiments with similar results. The densitometric analysis indicates elevated lysine acetylation in larvae cultured in the presence of VPA. The background staining observed in Western blots with anti-acetyl lysine antibody reflects the necessity for long time exposures for the visualization of acetylated lysine, likely indicating that the acetylated GFP::MDT-28 constitutes a minor proportion of the total GFP::MDT-28. The animals were synchronized by starvation after hatching and incubated for 4 hours with or without addition of bacterial food.

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 (Figures 12 – 15). This was apparent on recorded FLIM images compared to controls already after 4 hr incubations with 1 mM and 5 mM VPA and at 24 hr incubation with 1 mM VPA (Figures 12 and 13). The results were confirmed densitometrically (Figures 14 and 15).

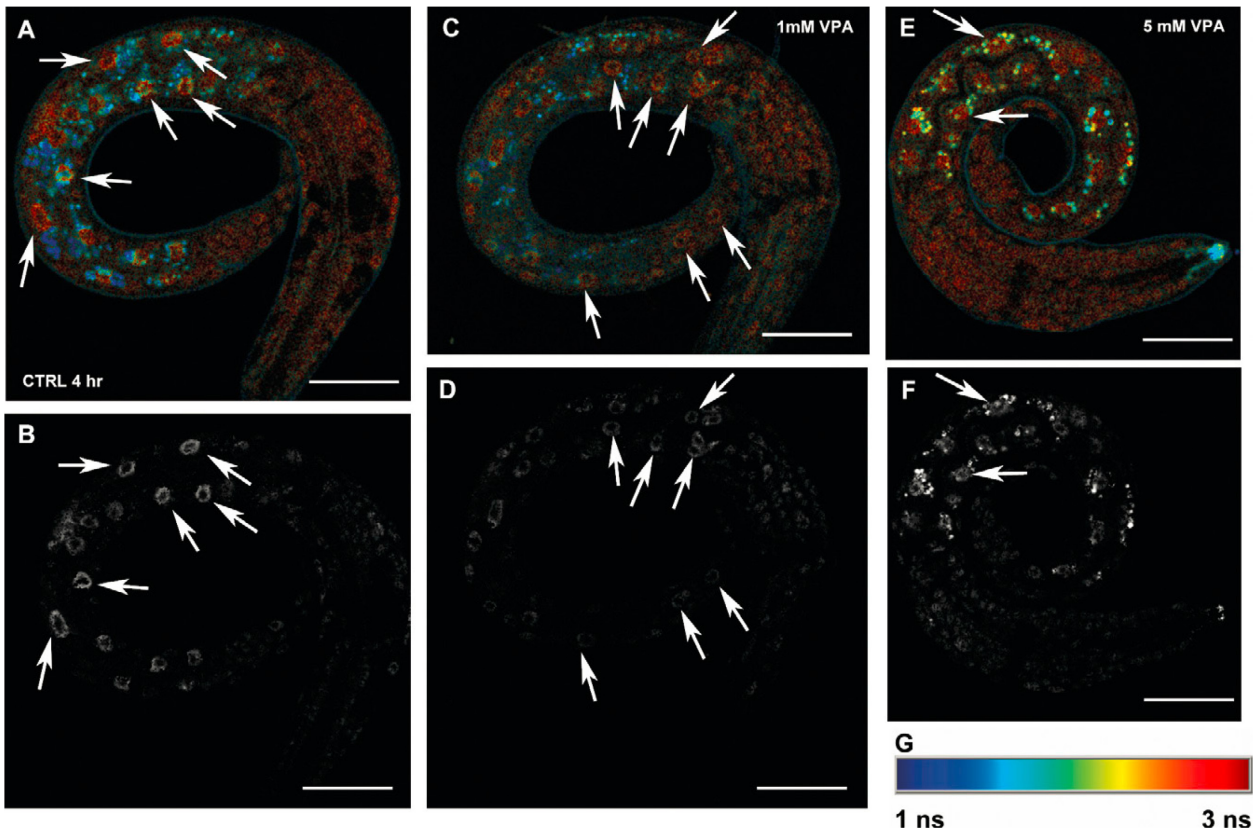

Figure 12. The effect of short-term (4 h) exposure to VPA on the intracellular distribution of GFP::MDT-28 in synchronized *C. elegans* L1 larvae. Panels A and B show a control L1 larva (no VPA), panels C and D an L1 larva after treatment with 1 mM VPA, and panels E and F a larva treated with 5 mM VPA. Panels A, C and E show computed false colour FLIM images. The reference table of average photon arrival time in FLIM images is shown in panel G. Panels B, D and F are the red

channel images corresponding to the signal from GFP only and show a visible decrease of GFP signal in the larvae incubated with 1 and 5 mM VPA. Bars represent 25 µm.

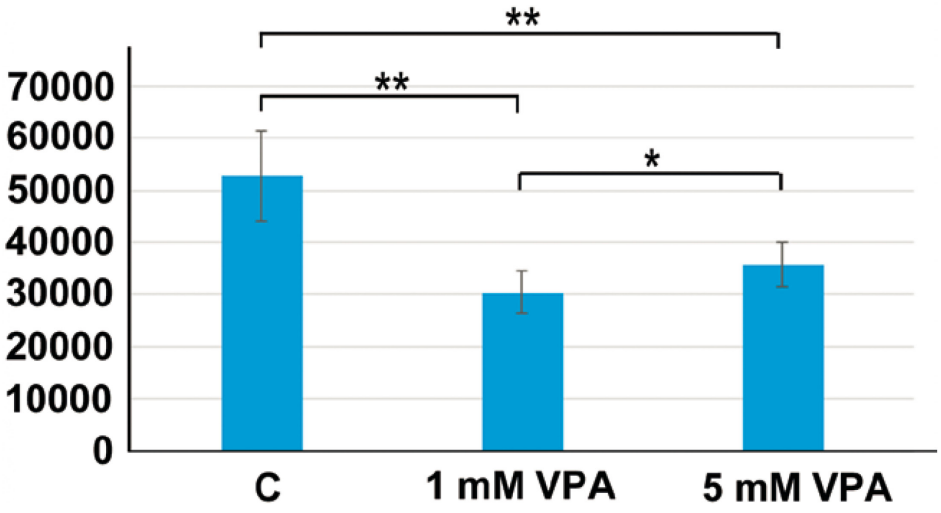

Figure 13. Densitometric analysis of GFP::MDT-28 FLIM signal in the nuclei of enterocytes of a control larva and larvae incubated with 1 mM and 5 mM VPA for 4 h. The GFP only signal, exported as red channel data obtained for individual nuclei, was analysed densitometrically using the ImageJ program. All clearly defined enterocyte nuclei (N = 35, N = 29 and N = 22, for control nuclei, nuclei of a larva incubated with 1 mM VPA and nuclei of a larva incubated with 5 mM VPA, respectively) were analysed. The values represent total pixel intensity. The P values of the *t*-test of paired sets, for which the null hypothesis is considered as no statistical difference between the two evaluated sets, are as follows: P = 1.61 × 10–8 for control versus 1 mM VPA, P = 3.67096 × 10–6 for control versus 5 mM VPA , P = 0 . 0 3 1 7 4 for 1 mM VPA versus 5 mM VPA. The results show a statistically significant decrease of GFP::MDT-28 in the nuclei of enterocytes treated with 1 and 5 mM VPA versus control nuclei (* < 0.05, ** < 0.00001).

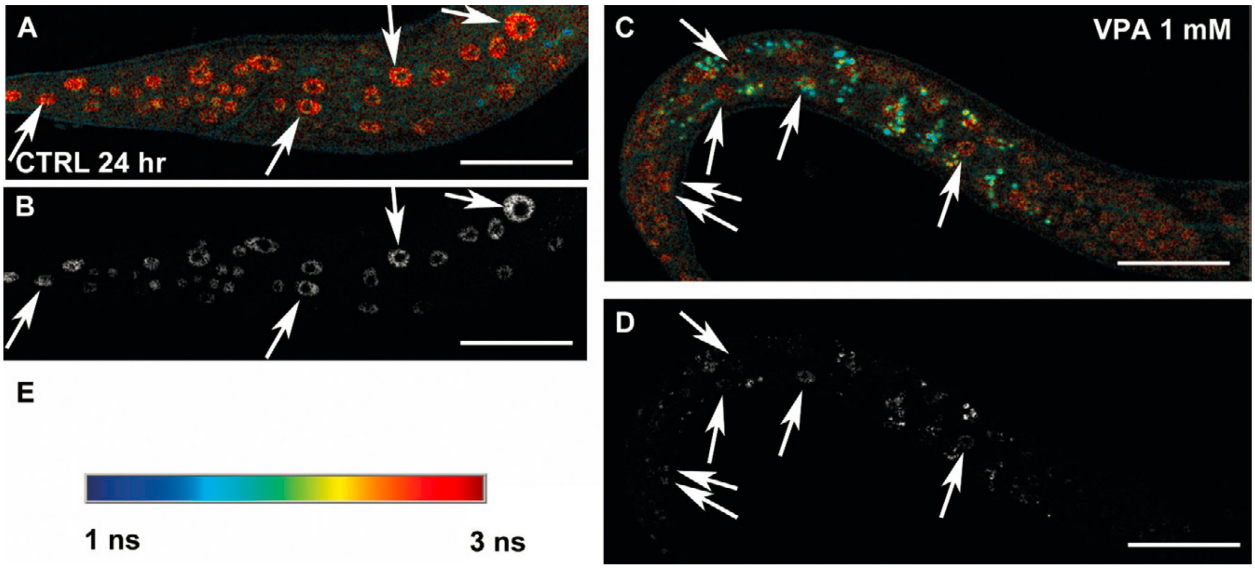

Figure 14. The effect of 24-h exposure of synchronized *C. elegans* starting from the L1 stage to valproic acid on the intracellular distribution of GFP::MDT-28. Panels A and B show a control larva and panels C and D a larva incubated with 1 mM VPA. Panels A and C show computed false color FLIM images of the overall signal. The reference table of average photon arrival time in FLIM images is shown in panel E. Panels B and D, derived from the red channel of false color FLIM images, display intracellular distribution of GFP::MDT-28 and show a visible decrease of the GFP signal in the larva incubated
with 1 mM VPA. Bars represent 25 μm.

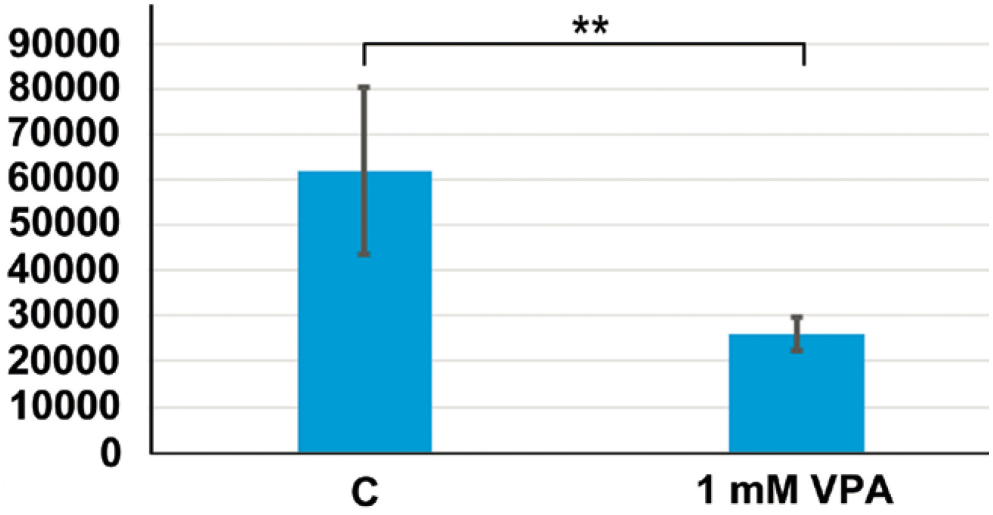

Figure 15. Densitometric analysis of GFP::MDT-28 FLIM signal in clearly defined enterocyte nuclei of a control larva and a larva incubated with 1 mM VPA for 24 h. The values represent total pixel

intensity. The P value of the *t*-test, for which the null hypothesis is considered as no statistical difference between the two evaluated sets, is P = 4.566 × 10–5. The result shows a significant decrease of the GFP::MDT-28 signal in the nuclei of enterocytes after 1 mM VPA treatment (** < 0.0001). N = 26 for control nuclei and N = 20 for nuclei of a larva treated with 1 mM VPA for 24 h.

Detection of intrinsically disordered regions in MDT-28

The study searched if intrinsically disordered regions can be detected by GlobPlot (http://globplot.embl.de/cgiDict.py) and are conserved in MDT-28. As expected, the bioinformatic tools detect 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 (Figure 16) 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).

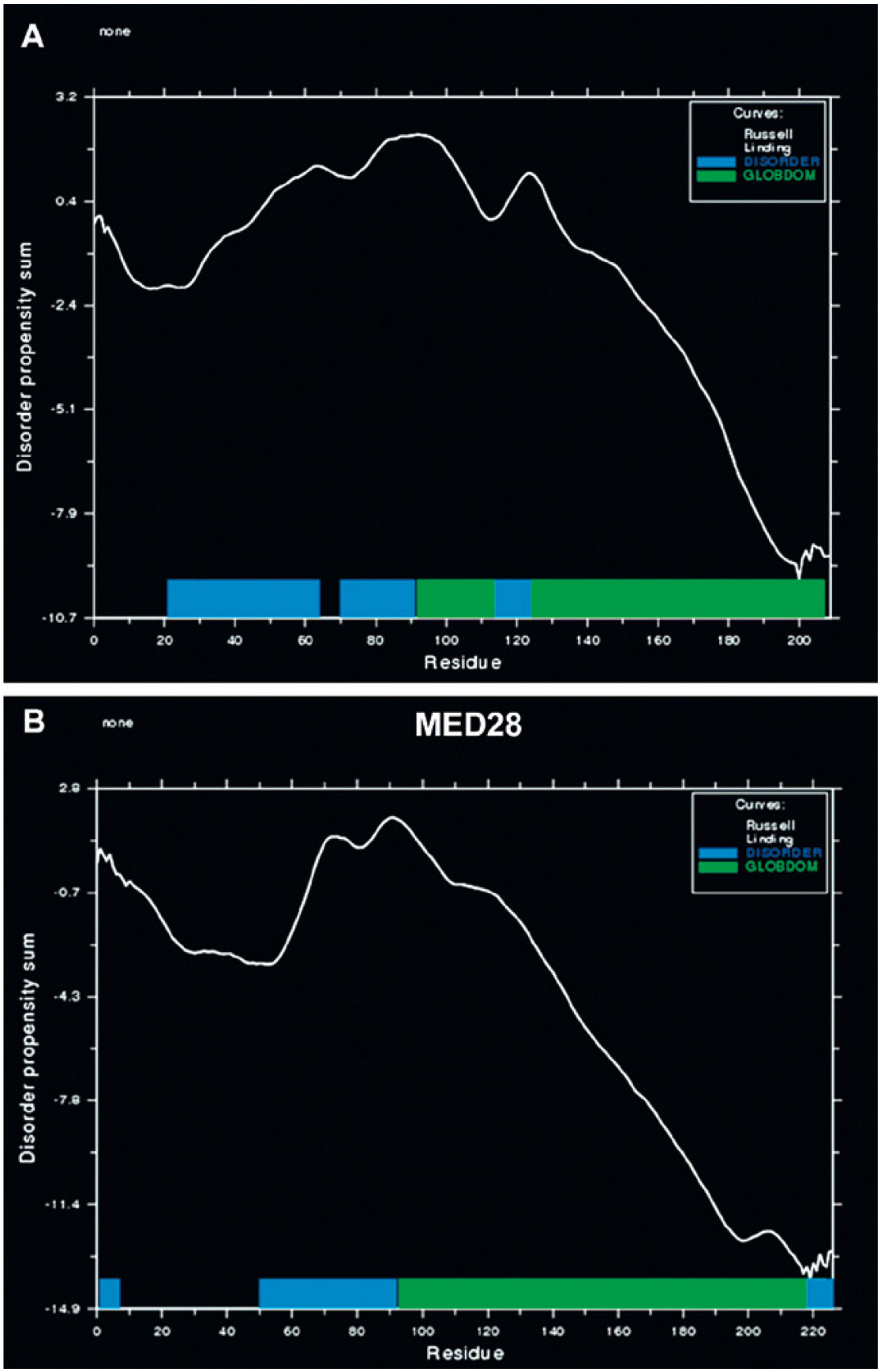

Figure 16. Schematic representation of intrinsically disordered regions in MDT-28 (panel A) and human MED28 (panel B). This schematic representation was done using GlobPlot

(http://globplot.embl.de/cgiDict.py). Both proteins show similar distribution of intrinsically disordered regions indicated by blue rectangles and structurally organized regions (detected as Globodomains) indicated by green rectangles. The globular domains are recognized as α structured domains in four models obtained by the SWISS-MODEL https://swissmodel.expasy.org/interactive/ahvMcA/models/, accessed on February 13, 2018) based on templates MED21, RalA-binding protein 1, Apolipoprotein E, and Apolipoprotein A-1. However, high fidelity of the models cannot be expected since no closer templates are available.

5.3 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 AA, Kaššák F, **Kostrouchová M**, Novotný JP, Krause MW, Saudek V, Kostrouch Z, Kostrouchová M. Perilipin-related protein regulates lipid metabolism in *C. elegans*. PeerJ. 2015 Sep 1;3:e1213. doi: 10.7717/peerj.1213. eCollection 2015; IF = 2.183)

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

First, W01A8.1 is approximately two times bigger than the mammalian MED28. Secondly, eventhough 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 W01A8.1 protein products localized in the cytoplasm on vesicular structures (Figure 17) subsequently identified as lipid droplets.

The experimental work that proves W01A8.1 as the sole orthologue of perilipins supports its reclassification but it is not critical for the thesis focus and is therefore only summarized below.

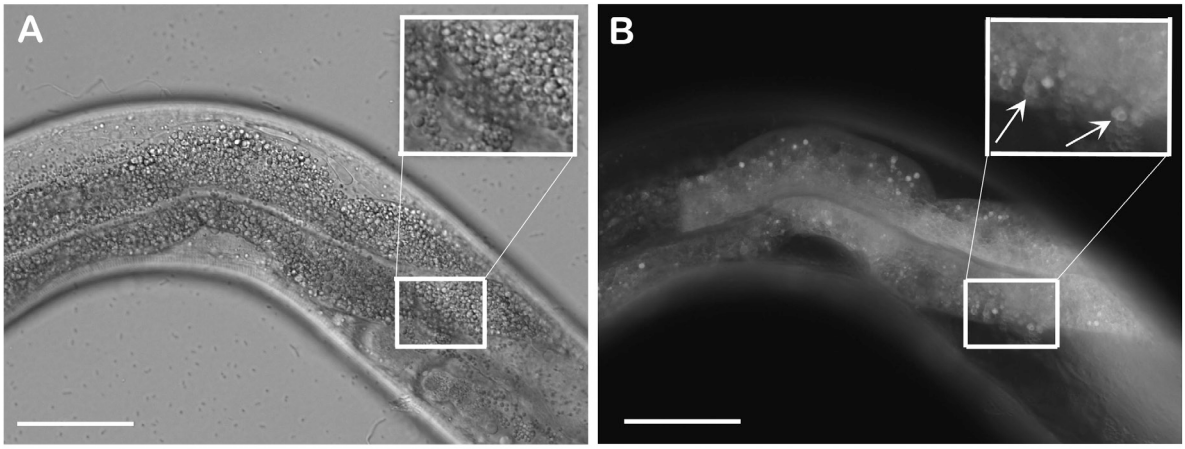

Figure 17. W01A8.1::GFP expressed from the edited gene in fluorescent microscopy. Panel A – Nomarski optics, panel B wide field fluorescent microscopy. W01A8.1::GFP shows predominant cytoplasmic localization accented around small cytoplasmic structures resembling lipid droplets. Lack of prominent nuclear expression of the tagged W01A8.1 contrasts with findings observed in the case of F28F8.5.

The proof that W01A8.1 is the nematode perilipin was obtained from 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.

Re-classification of W01A8.1 and classification of F28F8.5 as the true MED28 orthologue

Based on these results F28F8.5 is classified as a MED28 orthologue and is denominated (with the consent of WormBase) MDT-28.

Since the once given name cannot be removed from bioinformatic databases, both sequences, W01A8.1 and F28F8.5 have MDT-28 in their description but W01A8.1 is commented that this is an error. Our publications (Chughtai et al. 2015) and (Kostrouchova et al. 2017) serve as the evidence for the correction of the re-classification of W01A8.1 as the nematode orthologue of perilipins and the new classification of F28F8.5 as the true MED28 orthologue in *C. elegans*.

6. DISCUSSION

6.1. 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) (Kostrouchova et al. 2017) but it is not the previously denominated protein W01A8.1 (Bourbon 2008) which is in fact the sole orthologue of mammalian perilipins (Chughtai et al. 2015). 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 (Bickel et al. 2009; Liu et al. 2014), 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 important levels in cell nuclei. PLIN-1 loss of function led to clear alteration of lipid containing structures (Chughtai et al. 2015). 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 (Kostrouchova et al. 2017).

According to WormBase (WS270), *F28F8.5* is organized in a hybrid operon meaning that it can be expressed under the regulation of the promoter of the first gene in the operon or under the regulation of its internal promoter that is localized immediately before *F28F8.5* coding sequence. The observed expression of F28F8.5::GFP from the extrachromosomal arrays carrying *F28F8.5::gfp* under the regulation of *F28F8.5* internal promoter proves the functionality of the internal promoter. Interestingly, the expression pattern is almost identical with the pattern observed in animals with edited *F28F8.5* into the form of *gfp::F28F8.5*. The experiments also show that edited GFP::F28F8.5 is able to support all vital functions of F28F8.5 and F28F8.5 tagged on its C-terminus is not dominantly negative for vital F28F85 functions at least at the observed expression levels.

The operon CEOP5444 in which *F28F8.5* is organized contains 4 genes, *F28F8.7*, *F28F8.9*, *F28F8.6* (*atx-3*) and lastly *F28F8.5*. F28F8.7 is highly homologous to human RCOR1 (REST Corepressor 1), a component of the BHC corepressor complex that represses transcription of neuron-specific genes in non-neuronal cells acting as a chromatin modifier. In the BHC complex, it serves as a recruitment factor, that imposes silencing across a chromosomal region. It plays a central role in demethylation of Lys-4 of histone H3 by promoting demethylase activity of KDM1A. It also protects KDM1A from proteosomal degradation. RCOR1 is a part of RCOR/GFI/KDM1A/HDAC complex that suppresses, via histone deacetylase (HDAC) recruitment, a number of genes implicated in multilineage blood cell development and controls hematopoietic differentiation (Ballas et al. 2001; Lee et al. 2005; Lunyak et al. 2002).

The third gene in the operon is *F28F8.9*, the orthologue of human ATX3 which is a deubiquitinating enzyme with a wide functional spectrum. It is involved in regulation of transcription, cytoskeletal events, myogenesis and degradation of misfolded chaperone substrates. It binds and shortens long polyubiquitin chains. ATX3 interacts with key

regulators of transcription and acts as a transcriptional repressor probably by several mechanisms which include direct histone-binding (Li et al. 2002). (Mao et al. 2005). The organization of *F28F8.5* in the operon CEOP5444 suggests a functional connection between three out of 4 genes of this operon in the regulation of gene expression.

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 (Kostrouchova et al. 2018).

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 (Nagulapalli et al. 2016).

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

Contrary to the majority of the Mediator subunits, MED 28 does not have a yet completely clear localization within the Mediator structure (Verger et al. 2019). 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 (Tsai et al. 2014).

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) (Kostrouchova et al. 2017). 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 (Walter & Blobel 1983; Tokmakov et al. 2012; Kostrouchova et al. 2017).

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 (Wiederhold et al. 2004) and shown to be functioning as a regulator of

MEK1-dependent cellular migration in human breast cancer cells (Huang et al. 2012a). Its proven interactions with cytoplasmic proteins suggest it may have a regulatory potential to connect cell structural states with the regulation of gene expression (Lee et al. 2006).

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 (Huang et al. 2012b). In plants, non-nuclear functions of Med12 were also documented (Huang et al. 2012b). The Arabidopsis Mediator subunits Med4 and Med15 were found to interact with non-nuclear proteins (Nagulapalli et al. 2016; Kumar et al. 2018). 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 (www.The human protein atlas, accessed on February 25, 2019).

Our findings of the conserved dual localization of the nematode and mammalian MED28 orthologues further support 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 (Grants et al. 2015; Grants et al. 2016).

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) (Wiederhold et al. 2004). Similarly, the burst through vulva phenotype is also likely to be connected with LET-60/RAS signaling (Ecsedi et al. 2015; Rausch et al. 2015). 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 knowdown of *egfl7* led to abnormal blood vessel develepment. 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* (Rossi et al. 2015).

6.2 MDT-28 is a dynamically acetylated protein

Bioinformatic analysis of F28F8.5 reveals potentially 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 (Kostrouchova et al. 2017). 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.

6.3 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. The protein p53 is acetylated

in its C-terminal part with consequences for its functions in apoptosis as well as gene expression. Acetylation of lysines at the C-terminus has been shown to be connected with elevated degradation of p53 (Rodriguez et al. 2000) and it is therefore possible that the absolute concentration of MDT-28 is also decreased by the treatment with VPA.

7. CONCLUSION

Based on the published results the proteins F28F8.5 and W01A8.1 are correctly denominated in databaases 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. The work showed that lysine acetylation of MDT-28 is regulated and is augmented by treatment with a known inhibitor of histone deacetylases, valproic acid. The 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.

8. 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.

9. REFERENCES

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10. LIST OF PUBLICATIONS

Major publications included in the thesis

Kostrouchová M, Kostrouch D, Chughtai AA, Kaššák F, Novotný JP, Kostrouchová V, Benda A, Krause MW, Saudek V, Kostrouchová M, Kostrouch Z. The nematode homologue of Mediator complex subunit 28, F28F8.5, is a critical regulator of C. elegans development. PeerJ 2017, 5:e3390 https://doi.org/10.7717/peerj.3390; IF = 2.118

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Other publications related to the thesis topic

Kostrouch D, **Kostrouchová M**, Yilma P, Chughtai AA, Novotný JP, Novák P, Kostrouchová V, Kostrouchová M, Kostrouch Z. SKIP and BIR-1/Survivin have potential to integrate proteome status with gene expression; J Proteomics. 2014 Oct 14;110:93-106. doi: 10.1016/j.jprot.2014.07.023. Epub 2014 Aug 1. IF = 3.888

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