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Author's summary of the dissertation

UNIVERZITA KARLOVA
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Evolučně zachovalé mechanismy regulace genové exprese jadernými receptory

Evolutionarily conserved mechanisms of gene expression regulation by nuclear receptors

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

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Abstract

Transcriptional regulation of gene expression in eukaryotes has evolved over millions of years. The regulatory pathways of nuclear receptors represent an evolutionarily ancient, but conserved mechanism with associated accessory proteins, many of them forming a functional nexus known as the Mediator complex involved in transcription. Despite the versatility of the pathway, e.g. through the adoption of new regulatory functions in phylogenetically more recent Metazoa, we hypothesise that the intrinsic potential of the NR-Mediator axis to directly translate a stimulus to a biological response is conserved across species, and additional regulation could also be achieved through secondary functions of its essential members.

To support the hypothesis, we assessed the ligand-binding capability of retinoic X receptor in *Trichoplax adhaerens* and provided evidence to support the concept that this capability was already present at the base of metazoan evolution.

With regards to the potential secondary functions, we took inspiration from previous research and identified the Mediator subunit 28 (MED28) as the only known member having documented nuclear and cytoplasmic dual roles, and thus possessing the potential to transmit signals from the cellular structural states to the nucleus. Due to the lack of significant sequence conservation and a robust experimental toolset, we chose to characterise the presumed MED28 orthologue W01A8.1 in *Caenorhabditis elegans*. Our results suggest that W01A8.1 is in fact, with a high degree of certainty, a member of the Perilipin family thus, unveiling the previously unknown Perilipin-dependent regulation of lipid metabolism in Nematoda. This effort led to the reannotation as PLIN-1 (PeriLIpiN).

Keeping in line with the hypothesis, we subsequently identified F28F8.5 as the most probable orthologue of MED28 in *C. elegans*, which also consequently led to its reannotation as MDT-28 (MeDiaTor).

The work showed that the Mediator subunit 28 is a conserved member of the Mediator complex, which has a potential to connect regulation of transcription with cytoplasmic events. Together with the conserved NR signalling, it supports the hypothesis that the general architecture of the NR-Mediator signalling axis has been conserved throughout evolution of Metazoa. Additionally, I argue and make a case for Perilipin having a potential indirect and direct role in the regulation of gene expression. This incentivises further research on 'Proteome' signalling as a general principle.

Abstrakt

Transkripční regulace genové exprese eukaryotních organismů se vyvinula během milionů let. Regulační cesta jaderných receptorů představuje evolučně starý, ale zachovalý mechanismus zahrnující asociované akcesorní proteiny, z nichž mnohé tvoří funkční strukturu známou jako Mediátorový komplex, který je účastný v transkripci. Hypotézujeme, že přes universalitu této cesty, v níž vnitřní potenciál NR-Mediátorové cesty zachovalý napříč druhy, přímo překládá regulační signál do biologické odpovědi zapojením nových adaptačních funkcí ve fylogeneticky novějších Metazoidních organizmech a přídavná regulace může být dosažena sekundárními funkcemi základních členů této regulace.

Pro podporu této hypotézy jsme studovali schopnost vazby ligandu retinoidního X receptoru ve vločkovci *Trichoplax adhaerens* a přinesli důkaz podporující koncept, že tato schopnost byla přítomna již na počátku metazoidní evoluce.

S ohledem na možné sekundární funkce, majíce inspiraci z našeho předcházejícího výzkumu, jsme identifikovali Mediátorovou podjednotku 28 (MED28) jako jediný známý člen s dokumentovanou duální cytoplasmatickou a jadernou funkcí a mající tedy potenciál přenášet signály z jaderných strukturních stavů do jádra. V důsledku chybění významné zachovalosti sekvence, ale dostupnosti experimentálních přístupů, jsme se rozhodli charakterizovat předpokládaný ortolog MED28, W01A8.1 v *Caenorhabditis elegans*. Naše výsledky ukazují, že W01A8.1 je s velkým stupněm pravděpodobnosti člen proteinové rodiny perilipinů, což odhaluje v hlísticích dříve neznámou regulaci lipidního metabolismu závislou na perilipinu. Tato práce vedla ke změně klasifikace proteinu PLIN-1 (PeriLIpiN).

Ve shodě s touto hypotézou jsme dále identifikovali F28F8.5 jako nejvíce pravděpodobný ortolog MED28, který byl také následně klasifikován jako MDT-28 (MeDiaTor). Práce ukázala, že mediátorová podjednotka 28 je zachovalý člen Mediátorového komplexu, který má potenciál spojovat regulaci transkripce s cytoplasmatickými jevy. Společně se zachovalou signalizací NR to podporuje hypotézu, že generální architektura osy NR-Mediátorové signalizace byla zachována během evoluce Metazoa.

Následně navrhuji koncept, že Perilipin také může mít přímou a nepřímou úlohu v regulaci genové exprese, přičemž uvádím podpůrné argumenty, které motivují k dalšímu výzkumu obecného principu signalizace cestou proteomu.

Introduction

Scope and aims of the work

It is an established concept that the mechanisms involved in the regulation of gene expression are highly conserved in eukaryotes. The general scope and aims of work were as following:

- To explore the basic conserved mechanisms that play an intrinsic role in modulating gene expression in metazoans.
- To identify protein(s) that could have the potential to integrate complex environmental, metabolic, structural and stress-related cues and to relay this vital information to the transcriptional machinery.
- To gain a basic mechanistic understanding of how such protein(s) would function in a cell-specific manner.

Premises, hypotheses and goals of the thesis

Nuclear receptors are highly conserved transcription factors that are, in contrast to the other type of signalling pathways, capable of directly translating ligand binding into the regulation of gene expression through the Mediator complex. Thus, they make a direct link between stimulus and biological response, this potential of the system eases evolution. This is exemplified by the expansion of NRs in phylogenetically more recent metazoan organisms, such as nematodes (284 nuclear receptors) and mammals like humans (48 nuclear receptors), and by the formation of specialised endocrine tissues in vertebrates.

Therefore, it can be **hypothesised** that this intrinsic evolutionary potential of the NR-Mediator signalling axis, which is governed by its core function, would be conserved throughout metazoan evolution. As the general pathway architecture probably needs to be maintained, further plasticity of the pathway could eventually be achieved through the acquisition of secondary function by its essential members.

Among the members of the Mediator complex, the subunit 28 is the only member with proven cytoplasmic as well as nuclear functions. This makes it a candidate example protein that can integrate signalling between cytoplasmic structures and regulation of gene expression.

However, a comparative sequence analysis suggested significant differences between known vertebrate MED28 subunits and the denominated nematode orthologue in *C. elegans*. This indicated either a possible divergent evolution of the MED28 subunit or the acquisition of the Mediator domain in an unrelated gene that led to the annotation as the MED28 orthologue in nematodes.

To support this hypothesis, we set the following goals:

1. To show that NR-ligand interaction is conserved at the base of metazoan evolution.

2. To characterise the denominated MED28 orthologue (W01A8.1) in *C. elegans*.
3. In the case of non-divergent evolution, identify the true orthologue in *C. elegans*.
4. If another orthologue would be identified, then to access potential overlapping roles.

Study reasoning, strategy and design

Firstly, we chose to study the NR complement in probably one of the most basal metazoans, *Trichoplax adhaerens*, to address the question, whether the ligand-binding capability of NRs already existed at the base of animal evolution (Srivastava et al., 2008). And thus, provide information about the potential of NR-Mediator pathway to directly translate stimulus to gene expression.

We then asked how could a highly conserved pathway be also highly plastic, i.e. have an ability to adopt new regulatory functions over time in the course of evolution. *C. elegans* model system presented an excellent opportunity due to its recent phylogenetically expanded NR complement (Kostrouchova and Kostrouch, 2015), yet a relatively preserved Mediator complex (Grants et al., 2015). Moreover, it is a robust biological model system.

In our previous research, we had looked at structurally restricted proteins (localised to a specific part of the cell) having the potential to transmit the cellular status (of that part the cell) to the nucleus and influence gene expression. Due to the central position of the Mediator complex in transcriptional regulation we searched for a member of the Mediator complex that may also possess a similar ability to act as sensor of cellular states and transmit this towards the transcriptional machinery. Until now, the only member known to be displaying such potential was the Mediator subunit 28 – MED28 (Beyer et al., 2007; Wiederhold et al., 2004; Huang et al., 2012). Furthermore, the originally annotated MED28 orthologue in *C. elegans* (W01A8.1) had not been studied. This prompted a basic comparative protein sequence analysis of W01A8.1 with other known vertebral MED28 orthologues. The analysis showed a minimal sequence homology (MED28 domain) and suggested a possible divergent evolution of the nematode orthologue. Nevertheless, we reasoned that although it is possible that the nematode orthologue diversified during evolution, it would require strong evolutionary pressure because such a change could hinder the primary function of an essential member of the Mediator complex despite contributing towards the plasticity of the Mediator complex. The characterisation W01A8.1 in *C. elegans* could point towards newly evolved mechanisms of gene regulation in nematodes.

However, other possibilities should also be considered in case W01A8.1 would not share the common characteristics of a MED28 orthologue, such as the existence of an undescribed true orthologue.

Lastly, upon the existence of a true MED28 orthologue functioning independently to W01A8.1, the assessment of W01A8.1 regarding the involvement in the NR-Mediator pathway would shed light upon potential overlapping roles.

Methods and Materials

Animal culture, strains, transgenic lines and genome editing

We used various strains of the nematode species *C. elegans*. As wild type animals, N2 (var. Bristol), were used unless otherwise noted and all strains were maintained as described by (Brenner, 1974). Some of the strains and transgenic lines were requested from other research groups and others created through CRISPR/Cas9 technology. The implementation of the technology for our experiments in *C. elegans* was based on (Dickinson et al., 2013; Dickinson et al., 2015; Dickinson and Goldstein, 2016).
Regarding *Trichoplax* culture, the animlas were cultured in Petri dishes containing filtered artificial seawater with a salinity of approx. 38–40 ppt. *Rhodomonas salina*, *Chlorella sp.*, *Porphyridium cruentum* and other non-classified algae, as well as aquarium milieu established in the laboratory by mixing salt water obtained from a local aquarium shop were used to maintain stocks.

Sequence analyses

The characteristic molecular signature of the DNA binding domain (C-X2-C-Xl3-C-X2-C-X15-C-X5-C-X9-C-X2-C-X4-C-X4-M) (Kostrouch et al., 1995) was searched on JGI database (http://jgi.doe.gov/). The appropriate gene model (protein ID 53515) was selected for alignment with other species and performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Protein domain characterization was performed with SMART – a simple modular architecture research tool (Letunic et al, 2014). Phylogenetic analysis was performed on RXR ClustalO alignment using PhyMLv3.1 (Guindon et al., 2010) implemented in SeaView v4.6.1 with a 100 bootstrap analysis and SPR distance computation.
Perilipin orthologues and W01A8.1 sequences were extracted from UniProt, NCBI and OMA (omabrowser.org) databases. Chordate and nematode sequences were aligned separately using the T-Coffee algorithm (Notredame et al., 2000) (server tcoffee.crg.cat) and submitted to PSI-BLAST (Altschul, 1997) (E-value inclusion threshold $<10^{-3}$, 5 iterations) and HHpred (Agarwal et al., 2008; Remmert et al., 2011) searches as implemented in MPItoolkit (toolkit.tuebingen.mpg.de). Repeat detection used HHrepID module in MPItoolkit. Alignments were displayed and analysed in Jalview application (*www.jalview.org*).
MED28 orthologue sequences were searched in UniProtKB (uniprot.org) and NCBI (ncbi.nlm.nih.gov) with BLAST, PSI-BLAST (Altschul, 1997), HHblits (Remmert et al., 2011) and HHpred (Söding et. al, 2005). The obtained mammalian and insect sequences were aligned using T-coffee (Tommaso et al., 2011), as well as PROMALS (Pei et al.,

2007; Pei et al., 2008) and secondary structure predictions performed with PSIPRED (Cuff and Barton, 2000; McGuffin et al., 2000).

RNA interference

For RNAi based knock-down we used either the microinjection (dsRNA is directly injected into the *C. elegans* gonads) or the feeding method (Ahringer, 2006). The feeding method uses a special strain of *E. coli* to produce dsRNA inside the bacteria guided.

Transcript quantification

For the determination of W01A8.1 and F28F8.5 transcripts, we used the Roche Universal probe library and primers designed with the ProbeFinder Assay Design Software. qPCR was run on LightCycler 2.0. An average of three sample cDNAs and three control cDNAs were analysed, all containing the same amount of RNA for RT for each experiment. The expression was normalized to *ama-1.*
The Droplet digital PCR based measurements were performed on a Bio-Rad QX100 Droplet Digital PCR System. ddPCR was used for accurate quantification of small amount RNA obtained from cultured *T. adhaerens.*

LipidTox staining

The lipid staining protocol was done as described by O'Rourke et al., (2009) with modifications. The final concentration of 1:1000 dilution of LipidTox was used.

Microscopy and Imaging

Fluorescence microscopy and Nomarski optics microscopy were done using an Olympus BX60 microscope equipped with DP30BW CD camera. For live imaging the worms were anaesthetised with levamisole and placed on a cover glass with a thin layer of 1-2% agarose.

Coherent Anti-Stokes Raman Scattering Microscopy (CARS)

The CARS microscopy was used for lipid analysis in living cells as published by Potcoava et al., 2014. The CARS images were acquired with a Leica TCS SP8 CARS system consisting of a TCS SP8 confocal microscope combined with a picoEmerald laser.

Confocal and Fluorescence-lifetime imaging microscopy

Confocal microscopy of live homozygous animals was performed using an inverted Leica SP8 TCS SMD FLIM system operated by Leica Application Suite X program. The GFP fluorescence was excited at a wavelength of 488nm and the emitted light was simultaneously recorded in two spectral ranges, the two-channel setup was used to help resolve between spectrally different autofluorescence and GFP fluorescence signals.

Protein synthesis and binding studies

Bacterial and in vitro protein synthesis

For the purposes of performing *in vivo* ligand-protein and protein-protein binding analysis we used standard commercially available synthesis systems. RXR form Trichoplax was expressed in bacteria Bl21 pLysS using the pGEX-2T vector system.
The same protocol was used to synthesise the F28F8.5 protein in bacteria i.e. F28F8.5 coding sequence was cloned into pGEX-2T vector and transformed into BL21 cells. The MDT-6 protein synthesis was performed by cloning the coding region of *mdt-6* into pTNT vector and expressed in the rabbit reticulocyte Promega TNT-system based on the standard manufacture's guidance. The in vitro transcribed protein was labelled using ^{35}S Methionine.
The Mediator subunit MDT-30's coding sequence was cloned into pET28a(+) vector and transformed into BL21 *E. coli* cells. The lysate from bacteria producing His6-MDT-30-FLAG was used directly or purified on GE HiTrap Chelating HP column. Proteins produced by the TNT system or bacterial lysates of bacteria transformed with FLAG labelled Mediator subunits were incubated with glutathione–agarose adsorbed with equal amounts of GST or GST-F28F8.5.

Radioactive 9-*cis*-RA binding assay

For estimation of specific binding, 200-fold-excess of either 9-*cis*-RA or AT-RA was used. The radioactivity of the GST-fusion protein and cleaved protein was measured using Packard Tri-Carb 1600TR Liquid Scintillation Analyser and Ultima Gold Scintillation Fluid. The fraction of bound ^{3}H-labelled 9-*cis*-RA and ^{3}H-labelled all-*trans*-RA was determined as a ratio of the bound radioactivity of precipitated GST-TaRXR/total radioactivity used at the particular condition (determined as the sum of bound radioactivity and the total radioactivity of collected wash fluids) in the absence of non-radioactive competitors or 200-fold-excess of 9-*cis*-RA and all-*trans*-RA in the case of ^{3}H-labelled 9-*cis*-RA and 40-fold-excess of non-radioactive competitors in the case of ^{3}H-labelled all-*trans*-RA (to compensate for the higher affinity of 9-*cis*-RA compared to all-*trans*-RA in binding to TaRXR).

Radioactive protein interaction assay

The basic principle was to incubate the radioactively labelled mediator subunits with GST-tagged F28F8.5. ^{35}S Methionine labelled MDT-6 was translated using the rabbit reticulocyte TNT-system. While the FLAG-labelled MDT-30 was expressed from pET28(+) in BL21 *E. coli*. Proteins produced by the TNT system or bacterial lysates of bacteria transformed with FLAG labelled Mediator subunits were incubated with glutathione–agarose adsorbed with equal amounts of GST or GST-F28F8.5. The resulting samples (labelled proteins bound to GST- or GST-F28F8.5) were separated by polyacrylamide gel electrophoresis. ^{35}S-MDT-6 was visualised by autoradiography and

subsequently, the gel containing radioactively labelled protein was localised using superimposed autoradiograms, excised and the radioactivity determined in the scintillation detector. FLAG-labelled MDT-30 was determined by Western blot using an anti-FLAG antibody and quantified densitometrically by ImageJ computer program.

Results

Summarised using the three main publications (Novotný et al., 2017; Chughtai et al., 2015; Kostrouchová et al., 2017).

Part I

Trichoplax adhaerens retinoid X receptor is highly conserved

SMART (a simple modular architecture research tool) analysis of the proposed RXR sequence in *Trichoplax* showed a zinc finger DNA binding domain and a ligand binding domain with E values $<10^{-40}$. Blast analysis of the zinc finger DBD and LBD revealed a sequence identity of 81% and 70% to human RXR alpha, respectively. Each of these domains contained the predicted molecular pattern characteristic for each one. The heptad repeat LLLRLPAL, proposed for dimerization activity (Forman and Samuels, 1990), as well as the LBD signature for 9-*cis*-RA binding Q-x(33)-L-x(3)-F-x(2)-R-x(9)-L-x(44)-R-x(63)-H (Egea et al., 2000) are present. From the eleven amino acid residues known to be critical for 9-*cis*-RA binding nine are conserved and the remaining two amino acids are substituted.

RXR of *Trichoplax adhaerens* has conserved ligand binding ability

The fusion protein was then purified and used directly for binding studies or cleaved by thrombin and eluted as TaRXR. The binding of ^{3}H-labelled 9-*cis*-RA or ^{3}H-labelled all-*trans*-RA was determined by measuring total bound radioactivity and the radioactivity displaceable by 200-fold excess of non-radioactive competitors. The experiments showed that TaRXR (cleaved version) or GST-TaRXR bind 9-*cis*-RA with high affinity and specificity which is consistent with the highly conserved LBD. The 9-*cis*-RA binding assay showed high affinity binding to GST-TaRXR with a saturation plateau from 5nM to 10nM. However, all-*trans*-retinoic acid was unable to show high affinity binding to GST-TaRXR.

9-*cis*-retinoic acid at nanomolar concentrations can induce gene expression of malic enzyme

To know if 9-*cis*-RA has an observable biological effect *in vivo*. We, searched for an orthologue of vertebrate L-malate-NADP+ oxidoreductase in *T. adhaerens* genome (an established reporter of the state of thyroid hormone dependent regulation (Petty et al., 1990)).

Droplet digital PCR showed an increased transcription of the predicted L-malate-NADP+ oxidoreductase gene after incubation of *T. adhaerens* with 9-*cis*-RA, but not

with all-*trans*-RA. In repeated experiments, we observed that the level of induction was higher at 9-*cis*-RA concentrations in the range of 1 to 10nM, than above 10nM. We also noticed that the level of the induction slightly varied based on the actual *T. adhaerens* cultures and the algal food composition of the *T. adhaerens* cultures.

Part II

W01A8.1 shows structural similarities to vertebrate Perilipins

A sequence alignment of Plin2 and 3 from two selected vertebrates has been compared with their nematode homologues. Although the sequence-to-sequence comparisons are not sufficient to unravel the sequence homology between vertebrate and nematode Perilipin, the similarity appears clearly in the profile-to sequence (PSI-BLAST) and profile-to-profile (HHpred) searches. We can conclude that vertebrate Perilipins and the nematode W01A8.1 protein show a high degree of statistical homology.
An alignment encompasses a substantial part of *C. elegans* and human sequences (e.g., 90% of W01A8.1 and 87% of Perilipin 2) and covers all three domains characteristic for perilipins (N-terminal PAT, imperfect amphiphilic 11-mer repeat (Brasaemle, 2007) and C-terminal four-helix bundle (Hickenbottom et al., 2004). As W01A8.1 and human perilipins appear to be the best mutual reciprocal PSI-BLAST and HHpred hits, W01A8.1 is a very good candidate for a *C. elegans* orthologue of Perilipin.

W01A8.1 protein is cytoplasmic and reside primarily on lipid droplets

The translational fusion constructs resulted in high levels of cytoplasmic proteins present in intestinal and epidermal cells on vesicular structures with the characteristic appearance of lipid droplets. This pattern of expression and cellular distribution was observed beginning at the three-fold embryonic stage and continued throughout development to adulthood. Transgenic animals were stained with the lipophilic reagent LipidTox. The translational GFP fusion protein reporters were localized at the periphery of fat droplets that were LipidTox positive.

Human Perilipins label identical compartments as W01A8.1 protein in *C. elegans*

We prepared transgenic *C. elegans* lines expressing human PLIN1, PLIN2 and PLIN3 fused to GFP and regulated by the W01A8.1 promoter. PLIN1::GFP and PLIN2::GFP were localized on spherical cytoplasmic structures primarily in gut and epidermal cells with identical appearance as W01A8.1 translational reporter GFP tagged proteins and *Drosophila* PLIN1::GFP expressed in *C. elegans* as reported by (Liu et al., 2014). PLIN3 expression was diffusely cytoplasmic and only faintly defined spherical structures. The structures clearly labelled with PLIN1::GFP and PLIN2::GFP were also positive in LipidTox staining. We conclude that W01A8.1 is localized on the same structures as human PLIN1 and PLIN2.

W01A8.1 knockdown alters the appearance of lipid droplets in early embryos and causes a reduction of brood size

We used RNAi done by germline injection and by feeding. W01A8.1 RNAi made by microinjections and feeding resulted in significantly smaller brood size, with approximately 30% less progeny. RNAi made by microinjections resulted in ~52% reduced progeny laid in the first 24 h after microinjections and after 48 h ~28% reduction in progeny laid compared to controls. dsRNA feeding caused the W01A8.1 specific group to produce ~30% fewer larvae compared to controls. RT-qPCR confirmed that feeding based knockdown resulted in approximately 45% decrease in *W01A8.1* transcripts.

Staining of adult hermaphrodites with LipidTox (after formaldehyde fixation) revealed larger lipid droplets in early embryos derived from adults inhibited for W01A8.1 compared to controls.

Targeted disruption of W01A8.1 results in early embryonic defects but not lethality

CRISPR/Cas9-mediated gene editing eliminated almost the entire coding region. Animals with the deleted W01A8.1 locus were able to reproduce normally. As was observed in W01A8.1 RNAi embryos, loss of W01A8.1 activity resulted in the formation of large LipidTox-positive structures that were clearly bigger than droplets observed in control embryos. These large lipid-containing structures were observable in live mutant embryos but not in wild type embryos using Nomarski optics. Developing embryos lacking W01A8.1 showed that these large lipid droplets are present in embryos during the early mitotic divisions and were localised around the nucleus. Staining with LipidTox (after fixation) confirmed the lipid content in the vesicular structures arranged around the dividing nucleus. These large vesicles persist through the two-cell stage, disappearing in most embryos with more than 6 cells. On fixed embryos stained with LipidTox, larger than wild type lipid droplets are visible until late embryonic stages, including three-fold embryos.

CARS microscopy clearly confirmed the formation of large lipid-containing vesicles in early embryos and allowed detailed analysis of the W01A8.1 null phenotype. CARS microscopy also confirmed the gradual increase of the size of lipid-containing structures during oogenesis, the sudden re-localisation of these structures to the periphery of the dividing nucleus in the first embryonic division, and the propagation of this phenotype, although with gradually diminishing appearance, throughout embryonic development. CARS microscopy detected this phenomenon also in wild type animals, although the size of lipid-containing structures was smaller making the phenomenon of the sudden re-localisation of lipid-containing structures less obvious than in W01A8.1 null embryos. In contrast to embryos, lipid-containing structures in intestinal and epidermal cells of adult W01A8.1 null mutants were smaller than lipid-containing structures in control animals.

Morphometric analysis confirmed that *W01A8.1* null mutant embryos contained larger lipid positive structures recognised by CARS microscopy that represent a larger total area, as determined by quantitating individual focal planes images. Morphometry revealed many small structures. There was a clear inverse relation in the number of large and small structures for embryos as well as for adult tissues. The analysis shows that inclusion of small structures into analysis does not significantly affect the results but affects only standard deviation of the particle size distribution indicating that the results are independent on the setting of the limit for the size of lipid-containing structures. The probability of the results was assayed using Student's *t*-test and found to be statistically significant as the probability of this result, assuming the null hypothesis (no difference between control and experimental sets) was less than 0.0001.

Part III

F28F8.5 identifies as the closest homologue of vertebrate Mediator complex subunit 28 in *C. elegans*

To identify the *C. elegans* homologue of MED28, we queried protein databases with curated SwissProt sequences from UniProtKB. They comprised several mammalian and insect proteins. The more sensitive profile-to-profile HHblitz and HHpred algorithms provided hits to a *C. elegans* annotated protein F28F8.5a and b with highly significant *E*-values. According to Wormbase (WS248), two protein isoforms are produced from the *F28F8.5* gene, isoform a with the length of 200 amino acids and isoform b that has a two amino acid insertion at position 20 of the N-terminal evolutionarily non-conserved region. The best results were obtained when pre-aligned vertebrate and insect MED28 homologues were used as a query in three iterations ($E < 10^{-48}$ and the probability of true positive >99.99%). When the pre-aligned nematode sequences homologues to F28F8.5 were used to query profiles of human or *Drosophila* sequences in reciprocal searches, MED28 proteins were obtained with equally significant scores. Searches in protein databases were limited to sequences from *Ecdysozoa* with *Insecta* excluded (conservative inclusion threshold $E < 10^{-6}$) in the first two iterations and then continued in the complete database of sequences from all species in the subsequent iterations, the final hits of F28F8.5 included human and *Drosophila* MED28. PSI-BLAST with *T. spiralis* query sequence in database limited to *Ecdysozoa* in the first two iterations provided both human and *Drosophila* MED28 and F28F8.5 in one run ($E < 10^{-8}$). We concluded from these searches that F28F8.5 is a homologue of MED28 and very likely its previously unrecognised orthologue.

F28F8.5 is essential for life and localises to the nucleus and the cytoplasm

To determine the intracellular localisation of F28F8.5, we edited the *F28F8.5* gene using CRISPR/Cas9. The arrangement used in our experiment employed a self-excising cassette (SEC) that was added after *gfp.* This strategy initially creates a

disrupted *F28F8.5* gene and a null allele that can be picked out by GFP expression alone, which is regulated by the endogenous promoter of *F28F8.5*. We discovered that only the heterozygous animals could be propagated as the homozygotes tagged with this method were sterile. The results suggest that *F28F8.5* gene is essential for life.

After the removal of the SEC from the edited *F28F8.5* gene, this was phenotypically visualised by the continuous GFP::F28F8.5 fusion protein expression and loss of the RoI phenotypic marker. The resulting endogenous locus, as designed, had an N-terminus GFP-tagged *F28F8.5* gene. We were able to maintain these as homozygous animals, demonstrating this edited allele is fully functional.

The GFP::F28F8.5 pattern was ubiquitous, both nuclear and cytoplasmic from embryos to adults. Prominent nuclear localisation was found in oocytes, zygotes, larvae, and adults. Cells with clear nuclear accumulation of GFP::F28F8.5 included epidermal, intestinal, pharyngeal, uterine and vulval muscle cells. The gonad expressed *gfp::F28F8.5* and mitotic as well as meiotic nuclei accumulated GFP::F28F8.5 protein.

Confocal scanning revealed a strong GFP signal in the nuclei and cytoplasm in all developmental stages of life. Due to the overlap of the emission spectra of autofluorescence (from gut granules) and GFP, we used Fluorescence Lifetime Imaging Microscopy (FLIM) to distinguish between the two. Autofluorescence is likely to produce a signal with a short fluorescence lifetime as opposed to GFP. The result was clear, autofluorescent structures were clearly detected while fluorescence from GFP::F28F8.5, with a longer lifetime, was detected in the germline, in oocytes and embryos and in most somatic nuclei of larvae as well as adult animals.

F28F8.5 interacts with Mediator complex subunits and regulates development

We expressed ^{35}S-labeled MDT-6, a known interacting partner, in rabbit reticulocyte lysate and assayed its binding to bacterially expressed GST-F28F8.5 or to GST only. We saw a strong interaction (~7.7-fold enrichment) between MDT-6 to F28F8.5. Furthermore, we assayed the interaction between GST-F28F8.5 and MDT-30. We expressed MDT-30 containing a FLAG sequence inserted at the C-terminus and a His6 sequence positioned at the N-terminus. After expression in bacteria and purification on a nickel column, we found that the MDT-30-FLAG bound F28F8.5 preferentially (~2.5-fold enrichment) in comparison to GST alone, as revealed by Western blot using an anti-FLAG antibody.

Several experiments using knock-down by RNAi and homozygotic disruption by CRISPR/Cas9 approach revealed that F28F8.5 is essential for proper development. The progeny exhibited embryonic and larval arrest and a range of less severe phenotypes, including defective molting, protruding vulvae that often burst, male tail ray defects and uncoordinated (Unc) movement. The defects were exacerbated in animals lacking both functional alleles of the F25F8.5 gene.

Discussion

Structurally localised proteins show pleiotropic interactions and influence gene expression

It was suggested and discussed in our previous work (Kostrouch et al., 2014) that NR conveyed signals are integrated with cellular status through the Mediator complex in Metazoa. This indicated a possible connection between proteome status and gene expression by SKP-1 and BIR in *C. elegans* through interaction with various nuclear and cytoplasmic proteins (e.g. transcription factors and cytoskeletal proteins). In this work a potential link between SKP-1 and BIR-1 was studied. These two proteins are co-expressed from one operon and whose loss of function phenotypes have been linked to the regulation of gene expression and development. SKIP is an ancient transcriptional cofactor which interacts with several transcription factors including nuclear receptors (Barry et al., 2003) such as Notch (Zhou et al., 2000), Wnt/beta-catenin (Wang et al., 2010), TGF beta and Smad protein complexes (Leong et al., 2001). Furthermore, it has also been identified as a component of splicing machinery in; yeast; mammals (Zhang et al., 2003); and plants (Wang et al., 2012), and functions in transcription activating and inhibiting complexes (Kostrouchova et al., 2002). In *C. elegans*, SKIP is essential for normal development and its loss results in multiple phenotypes including larval transition and molting that is dependent upon NHR-23 (Kostrouchova et al., 2002). For some reason in *C. elegans*, SKIP (SKP-1) is co-organised in an operon together with a mitotic and microtubule organising protein BIR-1, a homologue of the vertebrate protein Survivin that is expressed predominantly in fast dividing cells and is up-regulated in most if not all human cancers (Li et al., 1998). Operons are polycistronic clusters of genes transcribed from a promoter at the 5' end of the cluster and by definition operons inherently lead to co-expression of co-organised genes, at least at the transcriptional level. Based on these presumptions the concept of the two proteins being functionally linked was conceivable. It had already been shown that both BIR-1 and SKIP are involved in gene expression regulation and development of *C. elegans,* furthermore, these factors act cooperatively in activating expression in a heterologous transfection system with thyroid receptor (Kostrouchova et al., 2003)

This work suggested a working model of how two proteins, one structural and another, a transcriptional cofactor could be involved in a protein regulatory network influencing gene expression at a transcriptional level. By searching for their interacting proteins using yeast two-hybrid screens it was observed that SKP-1 and BIR-1 interact with a wide variety of partially overlapping categories of proteins but not directly with each other. The regulatory potential of BIR-1 was experimentally visualised using a short time overproduction of BIR-1 in synchronised *C. elegans* larvae and by a whole proteome differential display. This hinted that elevated levels of BIR-1 project to immediate whole proteome changes. For the purposes of validation, the concept that certain proteins

which are known to be structurally bound such as ribosomal proteins (RPS3 and RPL5), non-muscle myosin and TAC-1 (a transcription cofactor and a centrosome-associated protein) were studied further. The results indicated that SKP-1 and BIR-1 are linked more than previously thought. They have the potential to link the proteome status with major cellular regulatory pathways including gene expression, ribosomal stress pathway, apoptosis and cell division. SKP-1 and BIR-1 could be regarded as proteome sensors. Based on the results obtained one could speculate that these pleiotropic protein interactions of SKP-1 and BIR-1 may be a part of a proteome regulatory network with the capacity to project proteomic states towards gene expression regulation. If such proteomic signalling would be observed as a more general mechanism by which proteome composition projects towards gene expression, then it may be suggested as a sort of 'Proteome' code. Such regulatory loops would possibly include proteins that are localised in specific cellular structures and when they are liberated or synthesised in excess of cellular needs then they assume their additional regulatory roles. In fact, such inhibition of gene expression was shown to be the auto-regulatory mechanism for RPL-12, which was shown to affect its own splicing most likely through a sensor affecting transcription (Mitrovich and Anderson, 2000).
These experiments as published in Kostrouch et al., (2014) lead us to search for more examples of proteins that may be involved in complex regulatory loops. As it is an established concept that cellular signalling through NRs and the Mediator complex represents evolutionarily conserved mechanisms that play a key role in regulating gene expression at the level of transcription. The Mediator complex functions by serving as a molecular bridge between DNA-bound transcriptional activators and the basal transcriptional machinery. Mediator complex co-activates NR-regulated gene expression by facilitating the recruitment and activation of the RNA polymerase II-associated basal transcriptional apparatus. Importantly, Mediator complex acts in concert with other NR co-activators involved in chromatin remodelling to initiate transcription of NR target genes in a multistep manner (Chen and Roeder, 2011). Based on these premises, it could be hypothesised that the individual protein components of the Mediator complex would allow one to explore the potential influence of many structurally restricted and/or localised proteins on gene expression.

RXR shows evolutionarily conserved mechanism of gene expression

An important aspect that wasn't investigated, until our data, was the functional level of NR conservation in basal metazoans. Investigating NRs in *C. elegans* as several pros and cons. One of the *contrā* of using *C. elegans* as a model for NR research is the fact that there are 284 NRs (Taubert et al., 2011) expressed by the genome, thus making the investigatory task complicated. Another *contrā* is the rather lack of high evolutionary conservation of many key vertebrate NRs in *C. elegans*. For instance, the worm lacks a single close orthologue of one of the major metabolic regulators, RXR, in its genome. Kostrouchova and Kostrouch, (2015) explain that although the most ancient

subfamily of NRs is the NR2 subfamily and all the members are most conserved among many phyla (e.g. diploblasts, vertebrates and mollusks), in nematodes the conservation is less apparent and one major founder, RXR, which is highly conserved between diploblastic species, vertebrates and mollusks, happens to be missing in *C. elegans*. Genetic analysis has revealed that *T. adhaerens* is one of the most basal metazoans known with a highly conserved set of NRs. This very concept presented an appealing opportunity. Until our data no published work had shown the functional conservation of RXR in *T. adhaerens*. Our data as published in Novotný et al., (2017) demonstrate the presence of 9-*cis*- RA binding RXR in Placozoa and argues for the existence of ligand regulated NRs at the base of metazoan evolution. The presence of functional nuclear receptors in *T. adhaerens* and their proposed regulatory network supports the hypothesis of a basic regulatory mechanism by NRs, which may have been subspecialised with the appearance of new NRs in order to cope with new environmental and behavioural challenges during the course of metazoan evolution and developmental regulatory needs of increasingly more complex metazoan species. Keeping with our published finding were the data provided by Reitzel et al., (2018).

W01A8.1 shows more structural similarities to vertebrate Perilipins than to MED28

As established through several previous publications that MED28 was an interacting partner with many other proteins including cytoskeletal proteins (Wiederhold et al., 2004). These potential properties made it an ideal candidate for our investigation. Our initial search for MED28 homologue in *C. elegans* was performed back in 2014, simply searching for MED28 in *C. elegans* databases yielded a positive result. The search yielded a gene sequence name W01A8.1 which had been curated as mdt-28 and had been described as an orthologue of mammalian MED28. It is perhaps clear that the Mediator complex being an essential part of the functional transcription process, would be conserved throughout eukaryotes. And there is sufficient data to support that claim, in fact, the structure and subunit organisation are also conserved between yeast and humans (Conaway and Conaway, 2011; Malik and Roeder, 2010). So, its existence is not really a surprise, in fact, its absence in nematodes would be much more astonishing. The review published in 2015 established a list of known mediator members in *C. elegans* (Grants et al., 2015). The authors established a list of the putative *C. elegans* Mediator subunits along with their mammalian orthologues, alternative names, sequence number and even the hypothetical location within Mediator, this was also based on the work by (Tsai et al., 2014).

A report by Zhang et al., 2012 focused on LD proteomics, they isolated and analysed LD resident proteins from *C. elegans.* In this large-scale proteomics analysis, it was revealed that many proteins with diverse functions are resident or associated with lipid droplets. Of great interest to us was that fact that W01A8.1 could also be found on LDs

and they also annotated the protein as a Mediator complex member protein involved in transcription.

However, we first, using basic comparative protein analysis, established that protein annotated as the *C. elegans* MED28 did show significant sequence homology to MED28 but rather showed close structural homology to another protein known to exist in other species as Perilipin. We then investigated the sequence alignment of *C. elegans* and human homologues of Perilipin which, however, did not appear visually very informative, but the underlying evolutionary conserved homology was statistically very significant. This notion has two issues firstly, it fundamentally challenged the pre-existing view of *C. elegans* not carrying a Perilipin homologue in its genome (Lee et al., 2014). Secondly, it raised a serious, but an exciting, question about the true homologue of MED28 in *C. elegans*. Furthermore, despite the statistically significant structural similarity of W01A8.1 to Perilipin the protein sequence analysed using Conserved Domain searches yielded results showing W01A8.1 containing both domains. This was not only apparent in our searches but also showed to be the case by other investigators. Vrablik et al., (2015) and Na et al., (2015) also investigated the *C. elegans* protein W01A8.1 and published findings at a similar time frame to ours. Vrablik et al., 2015 state that even though the protein shares sequence similarity to the transcriptional mediator complex protein 28, it also contains an N-terminal domain similarity to pfam 03036, which is a conserved domain associated with the Perilipin family.

Based on the knowledge at hand one could potentially postulate a dual role of the protein W01A8.1 in *C. elegans* i.e. a transcriptional role, associated with the MED28 superfamily domain, and a role as a structural regulator of LD, associated with the Perilipin domain.

Na et al., (2015) identified W01A8.1 as one of the major LD resident proteins which they referred to as a component of the multi-subunit transcriptional mediator complex. Nonetheless, they also went a step further and determined the regions of W01A8.1 protein responsible for its LD targeting. The authors demonstrated that truncation mutant peptides containing amino acids 211 through 275 of W01A8.1 formed ring structures around LipidTox-stained LDs in CHO K2 cells.

There could be many possible explanations for the apparent dichotomous evolution at least at the level of the primary sequence of W01A8.1 i.e. containing conserved domains of two different proteins. Looking from an evolutionary point of view at Perilipin, it is a scaffolding protein which could have resulted in co-evolution of interacting domains and divergence of non-docking sequences, thus the function could be conserved even with limited amino acid conservation across species. This evolutionary plasticity is apparent in the alignment of the human Perilipin paralogues where only the knowledge of the three-dimensional structure enabled observations of the similarities in the C-terminal domains (Hickenbottom et al., 2004). It is possible to

imagine that the nematode sequences have diverged beyond the point where pairwise comparisons used in routine searches can reveal homology, hence the difficulty in identifying the nematode orthologues. Only rigorous statistical analysis of the hidden Markov profiles of a great number of diverse sequences made it possible to identify the conserved domain composition. However, one should not rule out potential association of W01A8.1 with the members of the Mediator complex or for that matter with the transcriptional apparatus.

The binding ability of Perilipins to LDs is conserved

We conducted several experiments that allowed us to better understand the enigma of W01A8.1. We first created several constructs with W01A8.1 fused to GFP at the C-Terminus. The first attempt was done using a plasmid-based extrachromosomal approach. Promoter and coding sequence of *W01A8.1a* isoform was cloned into a GFP expression vector and injected into the gonadal tissue using the standard protocol. However, this resulted in embryonic lethality and we were unable to obtain a viable GFP expressing line. These effects were most likely attributable to the overexpression of the gene, often associated with plasmid-based extrachromosomal arrays. We then used another approach known as Fusion PCR to localise W01A8.1. With this method, we were successfully able to obtain a viable GFP line. This experiment revealed, for the very first time, a distinct pattern of cytoplasmic distribution which was at the time not known. W01A8.1 formed ring-like structures in the cytoplasm and the signal could be observed in almost all tissues. It was quite clear that these ring-like structures were most likely intracellular fat/lipid droplets as we were aware of the statistical similarity of W01A8.1 to Perilipins beforehand. To prove that these structures were truly we LDs we used Lipid staining dyes to chemically label them.

Lipid labelling in *C. elegans* can be rather tricky and can produce varied results depending upon the method used. Commonly used dyes include Nile red BODIPY-labelled fatty acids. Both stains have been used as vital dyes in living animals including worms. Nile red and BODIPY-labelled fatty acids stain living worms when the dyes are mixed with their food, *E. coli* bacteria (Ashrafi et al., 2003). However, there were many discrepancies found in several publications regarding the validity of truly representing the lipid storage in living *C. elegans*. These discrepancies led to a landmark discovery by O'Rourke et al. (2009), who showed that when Nile red is used as vital dye, it accumulates into lysosome-related organelles (LROs). And Nile red-positive LROs do not contain the major *C. elegans* lipid stores. Moreover, they show that the major lipid stores are contained within independent specialised neutral lipid-containing vesicles (LDs). In addition, they also revealed that Nile red and BODIPY-labeled fatty acids, when used on fixed *C. elegans*, are able to stain the neutral lipid-containing vesicles. The authors also recommend fixative-based dyes like LipidTOX and Oil Red O to localise lipids in *C. elegans*.

When fixed and stained W01A8.1::GFP worms that clearly revealed our prediction that W01A8.1 encapsulates neutral lipid-containing compartments (LDs). The expression was detectable in almost all tissues of the worm as well as embryos. Despite the fixation and staining protocols, it was very difficult to achieve complete penetrance of the fixative and/or stain in all worms and its tissue. This was very often the case for embryos, which are covered with the infamous chitin layer, making the staining process notoriously unreliable. Moreover, several fixation artefacts were very often visible. Sometimes we observed very large coalesced LipidTox stained fat-containing structures that were not visible in living worms before fixation. Regardless of the fixation artefacts, it became clear to us that W01A8.1 is a protein that is abundantly present on lipid-containing structures that were positively stained with LipidTox and also Oil Red O. To our knowledge this was the very first published direct evidence that W01A8.1 is surrounding LDs in *C. elegans.*

We went a step further to check if Perilipins from other vertebrates could also label the LDs in *C. elegans*. Another group ran similar experiments and expressed *Drosophila* PLIN1::GFP in *C. elegans* (Liu, et al., 2014). Their experiments were able to show that Drosophila Perilipin was able to localise to LDs in *C. elegans*. We wanted to see if this would also be the case for human Perilipins. We decided to investigate with Perilipin 1, 2 and 3 each marked with GFP using a natural promoter of W01A8.1. We used an extrachromosomal plasmid-based approach. The data gathered was actually consistent with what is already known about human Perilipins in human cells. Perilipin 1, when expressed in *C. elegans*, localised to the same LipidTox staining compartment. However, we observed that there were a significantly higher number of LDs and despite being easy to select, the line was not easy to maintain. This may be again attributed to potential toxicity of human Perilipin interfering with normal lipid metabolism. Perilipin 2 showed a similar pattern but was easier to maintain and did not lead to an increased number of total LDs. Perilipin 3 showed a dramatically different expression profile and was primarily cytoplasmic, which is actually analogous to the findings in mammalian cells (Itabe et al., 2017). One thing of note is the fact that these experiments were conducted on a wild-type background with normal W01A8.1. Overall our data and the data reported by Liu et al., (2014) implied that LDs found in *C. elegans* are, at least from a Perilipin binding ability point of view, similar. Clearly, Perilipin's position on the surface of LDs is a critical element in enabling it to regulate lipolysis, so it is not surprising that this targeting is evolutionarily conserved in all cell types in which Perilipins are present and this is a very suggestive clue in line with the notion of W01A8.1 being a Perilipin homologue.

Concerning the targeting sequence of Perilipin, Rowe et al., (2016) hypothesised that initial detection of, and interaction with, the LD surface by the 11-mer repeats triggers the 4-helix bundle to unfold and anchor the protein to the LD, although this proposed sequential model has yet to be formally proven. Their data highlights the importance of

amphipathic helices in detecting specific phospholipid membrane environments, which in the case of Perilipins means that they are ideally placed to precisely coordinate lipid release from droplets. However, protein truncation data presented by Na et al., (2015) show the amino acids 211 through 275 of W01A8.1 are responsible for the targeting to LDs. Based on our alignment, this region only partially overlaps with the 4-helix bundle. Therefore, it could suggest that although Perilipins from other species are able to bind LDs in *C. elegans*, the evolution of the binding ability of W01A8.1 could be different and may even implicate other evolutionary pressures acting upon W01A8.1 such as a potential transcriptional role, which is suggested by the presence of a MED28 superfamily domain.

W01A8.1 deficiency in embryos is reminiscent of Perilipin deficiency in other species

We employed RNAi and CRISPR/Cas9 mediated deletion approaches and looked for phenotypes. From the body of knowledge at hand regarding Perilipin from other species, it was difficult to predict if W01A8.1 would be lethal or not. In mammalian systems there are five Perilipin members, therefore, it is not easy to truly assess the viability as many Perilipins have redundant functions. Although deletion mutants are viable one cannot forget about the possible compensatory mechanisms taken over by other members of the family (Bulankina et al., 2009). Data from *Drosophila* suggest that Perilipin deletion mutants are viable but proper lipid homeostasis in flies lacking (Beller et al., 2010; Bi et al., 2012).

We conducted RNAi experiments using the injection and feeding methods, one caveat of RNAi in *C. elegans* is achieving consistent knockdown throughout each worm and between experiments. Using both methods we were able to see some phenotypes despite the fact that only a 45% knockdown could be quantified using RT-qPCR for the feeding experiments. We observed a reduced brood size of 30% fewer progeny produced and clumped lipid droplets in the germ line. With CRISPR/Cas9 we obtain a null mutant. The morphological phenotype of the mutant was consistent with what was observed by RNAi, however, we didn't observe any significant difference in brood size. Our initial explanation for the discrepancy was ascribed to the fact that RNAi induced an acute deficiency which was harder to compensate for in comparison to null mutants. Additionally, our morphological findings are in line with other publications (published at a later date to ours) from Na et al., (2015) and Vrablik et al., (2015). Due to experimental limitation of lipid assessment in *C. elegans* when using fixative dyes, we turned to Coherent anti-Stokes Raman Scattering (CARS) microscopy for a much more precise phenotypical analysis.

Analysis of lipid-containing structures, using CARS, in developing embryos and in adult tissues suggested that W01A8.1 protein isoforms could differ in embryos and adult tissues. Detection of lipids *in vivo* showed that the depletion of the Perilipin homologue

affects the intracellular distribution of lipid droplets, which is in agreement with the role of Perilipin homologue LSD2 (PLIN2) in the movement of lipid droplets in *Drosophila* (Welte et al., 2005). The characteristic aggregation of lipid-containing structures around the embryonic nuclei clearly detected in *C. elegans* embryos by CARS microscopy are reminiscent of lipid droplets found reported in hepatitis C virus (HCV) infected cells (Boulant et al., 2008). The work of Welte et al., (2005) and Boulant et al., (2008) provided the much-needed insight into the potential mechanism behind some of the observed phenomena, at least with regard to the embryonic phenotype. Boulant et al., (2008) show conclusively that attachment of the hepatitis C core protein to LDs induces aggregation around the nucleus in HCV-infected cells. This redistribution is accompanied by a loss in Perilipin 2 from the surface of LDs as increasing amounts of the core protein associated with the LDs.

Welte et al., (2005) showed that Perilipin is specifically required for the regulated transport of lipid droplets in *Drosophila*. This requirement can be seen at the level of the whole fly by phase contrast microscopy. In wild-type *Drosophila* embryos, lipid droplets are transported toward the centre of the syncytial embryo just prior to cellularisation (during phase II), which results in increased translucence (clearing) of the peripheral cytoplasm. However, in Perilipin mutant embryos, the peripheral cytoplasm fails to clear completely and the embryo remains mildly hazy throughout embryogenesis. They find that the Perilipin is required to regulate the motor-driven motion of lipid droplets in *Drosophila* embryos. In the absence of Perilipin, droplets move in a vigorous pattern, but their motion cannot be regulated. Thus, the phenotypical changes observed in embryos after knockdown and knockout of W01A8.1 are ascribable to the functions of Perilipin.

W01A8.1 affects lipid content differently in somatic and embryonic tissue

For W01A8.1 to be a Perilipin orthologue in *C. elegans* it also has to have an effect on lipid content and not just distribution.

CARS microscopy was used to quantify the total fat content in somatic and embryonic tissue. Our data strongly suggested that in somatic tissue there was approximately 30% less fat compared to wild-type controls and the situation was reversed for the embryonic tissue where there was approximately 30% more fat. The finding of the somatic tissue TAG content was also confirmed by Na et al., (2015), who quantified TAG/total protein content of L4 larval stage (without embryos). Although they claim that the difference was minor, one has to note that L4 stage worms already have an almost mature germ line with developing unfertilised eggs. We believe that could be contributing to some of the TAG measured as in our CARS imaging one can also observe the increased fat content in developing egg cells.

If one presumes that W01A8.1 is Perilipin then it is possible to start to explain the somatic phenotype in context of lipolysis. Different Perilipins behave differently in mammalian systems so to have a direct correlation can be tricky. Generally, Perilipins

effectively protect stored triacylglycerides from lipolysis under basal conditions, however, when hormonal signals activate PKA, Perilipins mediate the increase in lipolysis. Deficiency of both Perilipins leads to a reduced fat content in mammalian cells. Their overexpression is linked to increased fat content (Sztalryd and Brasaemle, 2017). With respect to our observed findings, we see in absence of W01A8.1 a reduced overall fat content. And although we didn't make specific experimentation on the overexpression phenotype we did observe enlarged LDs, when we inadvertently overexpressed the W01A8.1 using the fusion PCR technique to create a translational fusion with GFP.

In general, these observations in somatic tissue after knockout and mild overexpression of W01A8.1 are better attributed to the function Perilipin than to MED28. Perilipins were also implicated in the regulation of lipophagy (Kaushik and Cuervo, 2016). Our experimentation also shows that *C. elegans* embryos lacking W01A8.1 have increased autophagic activity – These experiments has been published as a Pre-Print article currently under review (Kaššák et al., 2019). This would be consistent with the role of Perilipins being protectors against lipophagy.

F28F8.5 is most likely the true orthologue of mammalian Mediator complex 28

Having fairly strong lines of evidence suggesting W01A8.1 as a Perilipin orthologue it was vital that we dedicated some of our efforts also towards finding an explanation for the missing role as a MED28 orthologue. Rather than beginning to look for MED28 properties of W01A8.1 we resorted to finding an alternative protein that could perhaps be the true MED28. Our experimentation of W01A8.1 had already shown us that despite containing a MED28 superfamily domain in the primary sequence it didn't show strong phenotypical attributes namely a presence in the nucleus as well as a developmental disorder in case of deficiency. By searching for another possible orthologue of MED28 it could absolve W01A8.1 for being the member of the highly conserved Mediator complex.

Although individual Mediator complex subunits were shown to be associated with specific functions (reviewed by Grants et al., (2015)), the function of the nematode orthologue of MED28 could not be studied since it has not been identified. MED28 has a special position among Mediator subunit proteins for its dual regulatory role, one as a Mediator subunit (Beyer et al., 2007) and the second, which is cytoplasmic, as a cytoskeletal related protein (Wiederhold et al., 2004; Huang et al., 2012).

Thinking it was unlikely that a MED28 orthologue would be absent in nematodes, we searched for it using the conserved features of MED28 orthologues from various phyla. Herein we identified a previously uncharacterised protein, F28F8.5, as the closest MED28 orthologue. F28F8.5 localised to both nuclear and cytoplasmic compartments in most, cells throughout development. The phenotypes that we observed in F28F8.5

knock-down and loss of function experiments overlapped with the EGFR regulatory cascade in *C. elegans*, especially the developmental defects of the vulva and of male-specific structures, male rays (Grants et al., 2015; Grants et al., 2016). Our observation of the expression of *F28F8.5* in male rays and the defective development of male-specific structures after *F28F8.5* RNAi support the cytoplasmic role of F28F8.5, that is in mammals mediated by Grb2 (Wiederhold et al., 2004). F28F8.5 contains a predicted SH2 binding site for Grb2 in the loop positioned in-between the two helices of F28F8.5, similarly as MED28 (identified using the site prediction tool Motif Scan http://scansite.mit.edu/motifscan_seq.phtml). The burst through vulva phenotype is also likely to be connected to LET-60/Ras signalling that also supports the conservation of the dual, nuclear and cytoplasmic functions, of MED28 homologues throughout the evolution of Metazoa (Ecsedi et al., 2015). F28F8.5 was also shown to have tissue-specific functions, as in the anchor cell where it is important for the regulation of anchor cell translocation across the basement membrane during the formation of the developing vulva (Matus et al., 2010).
Our data showing the binding of previously experimentally identified members (MED6 and MED30) in *C. elegans* with F28F8.5, truly provides sufficient evidence that F28F8.5 is the real orthologue of mammalian MED28. Furthermore, it absolves W01A8.1 from a very important task of being the highly conserved putative transcriptional regulator.

W01A8.1 influences the lipid droplet surface area

Having W01A8.1 exonerated from a potential orthologue of mammalian MED28 one can begin to analyse W01A8.1 in context of the whole lipid droplet being part of the super complex regulatory network of a cell.
The cellular cytoplasm is a congested space with thousands of proteins, RNA molecules, metabolites, etc swimming among several organelles. Zhang et al., (2012) and others have shown that many proteins from various functional categories can be found on LDs. Because many proteins can find refuge on the LD surface, the area can get crowded. This phenomenon is known as macromolecular crowding. Kory et al., (2015) proposed that the unusual organelle structure of LDs can result in protein crowding. They claim that macromolecular crowding plays a major role in determining LD protein composition. As a mechanism, protein crowding may be useful to cells, as it allows for the regulation of protein composition at the LD surface under dynamic conditions. For instance, protein crowding may dictate which proteins bind to LD surfaces during LD expansion versus shrinkage. They say that during lipolysis or shrinkage the surface gets crowded and only proteins with high affinity could stay on, while others with weaker affinity to the LDs are essentially pushed out of the ever-decreasing space. They also suggest that Perilipins might serve such a crowding-related regulatory function. They demonstrated that *Drosophila* Perilipin has a high binding affinity for LDs and is efficient in competing other proteins off the LD surface. Perilipin proteins might, therefore, increase the stringency of proteins binding to LDs, effectively limiting binding to those

proteins with relatively high affinity, thereby regulating the LD protein composition through a type of molecular proofreading.

We also observed the effects of W01A8.1 on the LD surface area. In null mutant embryos of W01A8.1, one can observe a relatively large LD surface area and the opposite is the case for the somatic tissue. This concept actually has many far-reaching implications on the indirect transcriptional regulatory ability of LDs or rather proteins affecting the size of LDs. So, one can argue that cytoplasmic events or in this case the size of lipid droplet can profoundly affect nuclear events. One major example of this is the implication of LDs in suppressing the activity of a transcription factor by keeping it out of the nucleus (Ueno et al., 2013). An LD protein Fsp27, also known as CIDEC, is expressed in adipocytes is known to promote fusion between droplets, causing the formation of a single droplet per cell (Jambunathan et al., 2011). Yeast two-hybrid interaction screens revealed the transcription factor NFAT5 (Nuclear factor of activated T cells) is a potential Fsp27 interaction partner. NFAT5 is cytoplasmic under hypotonic conditions and translocates to the nucleus upon hypertonic stress to activate osmoprotective genes (Aramburu et al., 2006). The research suggests that Fsp27 is able to sequester NFAT5 in the cytoplasm and interferes with its nuclear trafficking. And since in adipocytes endogenous Fsp27 is associated with lipid droplets, this interaction would retain NFAT5 at the droplet surface, something that remains to be demonstrated directly.

There are also other very relevant examples of LD surfaces as protein depots which have the ability to affect nuclear events. A *Drosophila* specific example is of histone storage on LDs. LD attachment of histones is mediated by a protein called Jabba, which functions as an anchor for histone. Jabba physically interacts with these histones, histones are absent from embryonic LDs in *Jabba* mutants, and the expression of Jabba in cultured *Drosophila* cells is sufficient to induce recruitment of histones to LDs (Li et al., 2012; Kolkhof et al., 2017). An enzyme CCT1 (CTP:phosphocholine cytidylyltransferase) also displays dramatic exchange between lipid droplets and nuclei. CCT1 is an enzyme that catalyses the rate-limiting step in the synthesis of the phospholipid phosphatidylcholine (PC) on the LD surface. In *Drosophila* cells, CCT1 is usually present in the nucleus, but under conditions in which cells synthesise new triglycerides and expand the hydrophobic core of droplets, CCT1 accumulates at the droplet surface (Guo et al., 2008; Krahmer et al., 2011).

In general, these examples are highlighting how cytoplasmic cues or rather metabolic cues in form of 'Proteome' signalling affect nuclear events, including transcription. As a point of note here, this notion of affecting nuclear events doesn't exclude other pathways, such as lipid signalling affecting transcriptional regulation (Georgiadi and Kersten, 2012).

Nuclear lipid droplets and nuclear Perilipin

LDs have been primarily considered cytoplasmic organelles. There have been ever increasing reports of the existence of LDs in the nucleus too. Briefly described LDs are known to be extensions of the phospholipid monolayer of the endoplasmic reticulum (ER), where the synthesis of neutral lipids occurs. Enzymes such as diacylglycerol O-acyltransferases (DGAT) form triacylglycerides (TAGs) and acetyl-CoA acetyltransferase form sterol esters (Hashemi and Goodman, 2015; Olzmann and Carvalho, 2019). Although many aspects of the process still remain unclear it is known that the initial budding process begins at the ER with the TAG synthesis machinery present on the ER membrane. After initial LDs (iLDs) are formed, a subset of them recruit enzymes via ER–LD membrane bridges and acquire the capacity to locally synthesise TAGs, converting them to expanding LDs (eLDs) (Wilfling et al., 2013; Farese and Walther, 2016). Expanding LD formation requires the Arf1/COP-I proteins to recruit TAG synthesis enzymes (Wilfling et al., 2014).

The rather inconstant observations of LDs in the nucleus have been around for a while dating back to the 70's (Hillman & Hillman, 1975), but recently more advanced methods (biochemical and electron microscopic) have also observed nuclear LDs (Layerenza et al., 2013; Uzbekov and Roingeard, 2013). But these findings are not consistent and not observed in all cell types. Recently, Ohsaki et al., (2016) used confocal and electron microscopy to investigate nuclear LDs in mammalian cells. They discovered that the LDs were located in the nucleoplasm and were not just an extension of the nuclear membrane. The authors also said that LD existence in the nucleus was scarce. Furthermore, they were able to find Perilipin 3 in the nucleus bound to the nuclear LD surface. Another exciting discovery made was the association of these nuclear LDs with nuclear bodies. They group found that promyelocytic leukaemia (PML) nuclear bodies labelled with anti-PML antibody co-localised with nuclear LDs. They showed that the PML-II protein played a critical role in nuclear LD formation. PML bodies have been implicated in a diverse range of functions including nuclear storage of proteins, post-translational modifications of proteins, direct involvement with transcription, and chromatin regulation (Lallemand-Breitenbach and de The, 2010).

Farese and Walther, (2016) discussed the implications and compared the cytoplasmic to nuclear LDs and they say that the nuclear LDs appear to be most closely related to expanding LDs found in the cytoplasm. Similar to cytoplasmic eLDs, nuclear LDs were found to co-localise with the TAG synthesis enzyme DGAT2 and its substrates, which should enable them to expand by locally synthesising TAGs. Nuclear LDs also have the CTP:phosphocholine cytidylyltransferase α (CCT) found on the surface for synthesising phosphatidylcholine (PC).

Liu et al., (2018) looked at one of the most commonly found proteins on *C. elegans* LDs. Apart from W01A8.1, these studies revealed that a large set of hydroxysteroid dehydrogenases (HSDs) are targeted to LDs. HSDs, which belong to the

superfamily of short-chain dehydrogenases/reductases (SDRs), are important enzymes involved in lipid metabolism and especially in steroid hormone metabolism. A member of the HSD family DHS-9 tagged with GFP was expressed in the intestine, and was found at high levels in the nucleus and at lower levels in the cytosol. The nuclear signal was diffuse but puncta were found in the nucleoplasm. Using advanced confocal microscopy (Airyscan) they showed clustered rings of GFP tagged DHS-9. They probed further and asked if these structures would co-localise with mCherry tagged W01A8.1. This experiment showed no co-localisation. However, they expressed W01A8.1::mCherry with a nuclear localisation signal (NLS) to see if the proteins would co-localise to the nuclear LDs. These transgenic animals showed a co-localisation of the W01A8.1::mCherry::NLS with DHS-9::GFP. This data provided tantalising insights into the complex regulatory link between metabolic processes and nuclear events. Studying nuclear LDs is a challenging task not only because of their scarcity but also due to their size. With regards to why W01A8.1 needed an NLS to localise to LDs could perhaps be explained, firstly, the protein comes in three isoforms and one would have to thoroughly investigate all three and secondly, it is possible that the real NLS (or rather hidden NLS) of W01A8.1 maybe actually shielded by the fluorescent protein attachment. Another landmark discovery by Gallardo-Montejano et al., (2016) actually showed that mammalian Perilipin 5 is involved in nuclear receptor-based transcriptional regulation. This recent exciting finding demonstrated in mouse cells Perilipin 5, under certain conditions, can translocate from the LD surface to the nucleoplasm and modulate gene expression. They showed that Perilipin 5's nuclear localisation is PKA-dependent. They also found that Perilipin 5 assembles into a complex with PGC-1α and SIRT-1. PGC-1 α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) is a transcriptional regulator important for mitochondrial biogenesis and function and SIRT-1 is a deacetylase that controls PGC-1 α's acetylation status and activity. Perilipin 5 mediates the activation of PCG-1α via its acetylation status. A protein known as Deleted in breast cancer 1 (DBC1) interacts with SIRT1 and inhibits its deacetylase activity. Their data suggested that Perilipin 5 is a regulator of the SIRT1-DBC1 complex during catecholamine stimulation and it influences the SIRT1 deacetylase activity in a manner that does not involve changes in total cellular NAD+ levels but rather through displacement of DBC1. All of this consequently affects target genes that promote fatty acid oxidation and mitochondrial efficiency. The dual role of Perilipin5 at the LD surface and in the nucleus was proposed to coordinate the release of FAs during lipolysis and their efficient usage by mitochondria. Keep with the theme of this thesis, one must also not forget to mention the C-terminal region of PGC-1α has been shown to play a vital role in interacting with the Mediator complex in particular with MED1 (Wallberg et al., 2003; Martínez-Redondo et al., 2015).

Conclusion and final remarks

In this body of work, several lines of evidence have been presented showing support of the hypothesis that NR-Mediator signalling pathway displays a high degree of evolutionary conservation. We provide results showing the ligand binding ability and biological response capacity of retinoic X receptor at the base of metazoan evolution. This is in line with the notion that the direct sensing ability is central to the NR-Mediator pathway.

Furthermore, we gathered data to prove that the previously denominated MED28 orthologue W01A8.1 is in fact involved in the regulation of lipid metabolism and is related to the Perilipin family of proteins that have been previously thought to be absent in nematodes.

Keeping in mind the potential regulatory functions of structurally localised protein I argue and make a case for W01A8.1 that, although it could not be localised in the nucleus, the presence of a Mediator domain could indicate a potential interaction with the highly conserved NR-Mediator axis.

Moreover, we were able to identify the true MED28 orthologue as F28F8.5 in *C. elegans*. Thereby showing that the Mediator subunit 28 is truly a conserved member of the Mediator complex and possesses the potential to connect cytoplasmic events to regulation of gene transcription. This supports the concept that the general architecture of the NR-Mediator signalling axis is conserved across species.

Additionally, our efforts have led to the discovery of Perilipin-dependent lipid metabolism in *C. elegans* and provide an incentive to study the concept of 'Proteome' signalling as a general principle, not restricted to only the members of the Mediator complex.

List of publications

Publications that are related to the dissertation

a) with Impact Factor

David Kostrouch, Markéta Kostrouchová, Petr Yilma, **Ahmed Ali Chughtai**, Jan Philipp Novotný, Petr Novák, Veronika Kostrouchová, Marta Kostrouchová, Zdeněk Kostrouch, SKIP and BIR-1/Survivin have potential to integrate proteome status with gene expression. Journal of Proteomics, Volume 110, 2014, Pages 93-106, ISSN 1874-3919, https://doi.org/10.1016/j.jprot.2014.07.023. (IF 3.9 - 2016).

Chughtai AA, Kaššák F, Kostrouchová M, Novotný JP, Krause MW, Saudek V, Kostrouch Z, Kostrouchová M. (2015) Perilipin-related protein regulates lipid metabolism in *C. elegans*.PeerJ3:e1213 https://doi.org/10.7717/peerj.1213 (IF 2.2 - 2016).

Kostrouchová M, Kostrouch D, **Chughtai AA**, Kaššák F, Novotný JP, Kostrouchová V, Benda A, Krause MW, Saudek V, Kostrouchová M, Kostrouch Z. (2017) The nematode homologue of Mediator complex subunit 28, F28F8.5, is a critical regulator of *C. elegans* development. PeerJ5:e3390 https://doi.org/10.7717/peerj.3390 (IF 2.2 - 2016).

Novotný JP, **Chughtai AA**, Kostrouchová M, Kostrouchová V, Kostrouch D, Kaššák F, Kaňa R, Schierwater B, Kostrouchová M, Kostrouch Z. (2017) *Trichoplax adhaerens* reveals a network of nuclear receptors sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution. PeerJ5:e3789 https://doi.org/10.7717/peerj.3789 (IF 2.2 - 2016).

b) without Impact Factor

Kaššák F, **Chughtai AA**, Kostrouchová M. 2019. Evolutionarily conserved roles of *Caenorhabditis elegans* perilipin in lipolysis. PeerJ Preprints 7:e27467v1https://doi.org/10.7287/peerj.preprints.27467v1.

Publications without relation to the dissertation

a) with Impact Factor

Gawish, A., **Chughtai, A.A.** & Eble, M.J. Strahlenther Onkol (2019) 195:383. https://doi.org/10.1007/s00066-018-1381-4 (IF 2.7 - 2016).

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