Univerzita Karlova

1. lékařská fakulta

Study program: Doctoral program in biomedicine

Study field: Biology and pathology of the cell

UNIVERZITA KARLOVA
1. lékařská fakulta

MUDr. Ahmed Ali Chughtai

Evolučně zachovalé mechanismy regulace genové exprese jadernými receptory

Evolutionarily conserved mechanisms of gene expression by nuclear receptors

Ph.D. Thesis

Supervisor: MUDr. Zdeněk Kostrouch, CSc.

Prague, 2019

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem řádně uvedl a citoval všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému mezi-univerzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

Prohlašuji, že odevzdaná tištěná verze práce a verze elektronická nahraná do Studijního informačního systému (SIS 1.LF UK) jsou totožné.

Praha, 06.08.2019

Ahmed Ali CHUGHTAI

Identifikační záznam:

CHUGHTAI, Ahmed Ali. Evolučně zachovalé mechanismy regulace genové exprese jadernými receptory (Evolutionarily conserved mechanisms of gene expression by nuclear receptors). Praha, 2019. 130s. Disertační práce. Univerzita Karlova v Praze, 1.lékařská fakulta, BIOCEV. Vedoucí práce Zdeněk Kostrouch.

Table of Contents

Relevant Abbreviations

9-*cis*-RA	9-*cis*-Retinoic acid
ABHD5/CGI-58	α/β hydrolase domain-containing protein/ Comparative Gene Identification
ACATs	acyl-coA cholesterol acyltransferases
ACS	acyl-coA synthetase
ACSL	Acyl-CoA synthetase
ADRP	PLIN2
AF-1/AF-2	Activation function 1 and 2
AGPATs	1-acyl-glycerol-3-phosphate acyltransferases
All-*trans*-RA/AT-RA	All-*trans*-Retinoic acid
AMP	Adenosine triphosphate
AMPK	Adenosine triphosphate kinase
APOs	Apolipoproteins
Arf1/COP-I	ADP ribosylation factor-1 / coat protein I
ARs	Androgen Receptors
ATGL/ATGL-1	Adipose tissue triacylglycerol lipase
ATGs	Autophagy-related proteins
BIR-1	Baculoviral IAP repeat proteins
CAR	Constitutive androstane receptor
CARS	Coherent anti-Stokes Raman Scattering
CBP	CREB-binding protein/p300
CCT1	CTP:phosphocholine cytidylyltransferase
CDKs	Cyklin dependent kinases
cGPDH	cytosolic glycerol-3-phosphate dehydrogenase
CHO K2	Chinese hamster ovary cells
CIDE/Fsp27	Cell Death Inducing DFFA Like Effector
CMA	Chaperone-mediated autophagy
COUP TF	Chicken ovalbumin upstream promoter transcription factor
CREB	cAMP response element-binding protein
DAG	Diacylglycerol
DBC-1	Deleted in breast cancer 1
DBD/LBD	DNA binding domain/Ligand binding domain
DGAT	Diglyceride acyltransferase
DHAP	Dihydroxyacetone phosphate
DHAP-OR	Dihydroxyacetone phosphate oxidoreductase
DHAPAT	Dihydroxyacetone phosphate acyltransferase
eLDs	Expanding lipid droplets
ELK1	ETS Like-1 protein Elk-1
ERR	Estrogen-related receptor
ERs	Estrogen Receptors

FLIM	Fluorescent Lifetime Imaging Microscopy
FXR	Farnesoid X receptor
GFP	Green fluorescent protein
GPATs	Glycerol-3-phosphate acyltransferases
Grb2	Growth factor receptor-bound protein 2
GRs	Glucocorticoid receptors
GRs	Glucocorticoid receptors
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
NFAT5	Nuclear factor of activated T cells
PAM	Protospacer-adjacent motif
PAP	Phosphatidic acid phosphatase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PIC	Preinitiation complex
PKA	Protein kinase A
PLINs	Perilipins
PML	Promyelocytic leukaemia
PPARs	Peroxisome proliferator-activated receptors
PXR	Pregnane X receptor
RISC	RNA-induced silencing complex
RXR	Retinoic X receptor
SKIP/SKP-1	Ski interacting protein
SMART	Simple Modular Architecture Research Tool
SMC	Regulating smooth muscle
SMRT/NCoR	Silencing mediator of thyroid hormone receptors/Nuclear receptor co-repressor
SREBP	Sterol regulatory element-binding protein 1α
SWI/SNF	SWItch/Sucrose Non-Fermentable
TAC-1	Preprotachykinin-1
TaRXR	Trichoplax adhaerens RXR
TR	Thyroid hormone receptor
TRAP	Thyroid hormone receptor-associated protein
USP	Ultraspiracle protein
VDR	vitamin D receptor
WED	Evolutionary divergent wedge domain

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.

Acknowledgements

I sincerely express my appreciation to all members of the laboratory involved in the exciting, thought-provoking and captivating adventure of science. I especially thank my supervisor Dr Zdeněk Kostrouch PhD for the extraordinary support and guidance. I would like also to thank all the colleagues/friends involved in the associated publications especially Dr Jan Philipp Novotný PhD for the unending encouragement and help with everything in life.

Lastly, I would like to thank all the members of my family for their love and support, especially my late grandfather who inspired me to become a scientist.

I would like to begin my thesis with an inspirational quote form Albert Einstein:

'Phantasie ist wichtiger als Wissen, denn Wissen ist begrenzt.'

Author's contribution to published work

The primary contribution was to the experimental work on W01A8.1/Perilipin orthologue in *C. elegans* – this included the theoretical reasoning, the design of the experimental strategy as well as the execution of most the experimental work relating to W01A8.1. I also participated on the experimental work concerning *Trichoplax adhaerens* RXR ligand-binding. I was responsible for the introduction, implementation, adaption and strategizing all CRISPR/Cas9 based experiments for W01A8.1/Perilipin and F28F8.5/MDT-28. And I was also actively involved in data analyses, interpretation of results as well as the preparation of all publications.

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 are responsible for integrating 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.

The underlying principles and background information: A review of literature

Regulation of gene expression

To start a step by step dissection of the complex regulatory mechanisms by starting at the source of all information i.e. at the DNA level. I will primarily focus on eukaryotic cell systems. In this section, I would like to highlight the major control points of gene regulation with regards to transcription mainly. Furthermore, I keep the focus on NR dependent transcriptional regulation and the role that Mediator complex plays as an effector.

Basic gene architecture and epigenetic control of gene expression

The very first layer of regulation is seen at the very structure of DNA itself and also the local environment it is sitting in i.e. the nucleus. The type of control is the so-called 'Epigenetic Control' of gene expression (Klose and Bird, 2006). This a relatively broad term representing a complex array of structural and chemical changes that are made to the DNA polymer as well as the protein molecules (e.g. histones) that the DNA molecule is wrapped around.

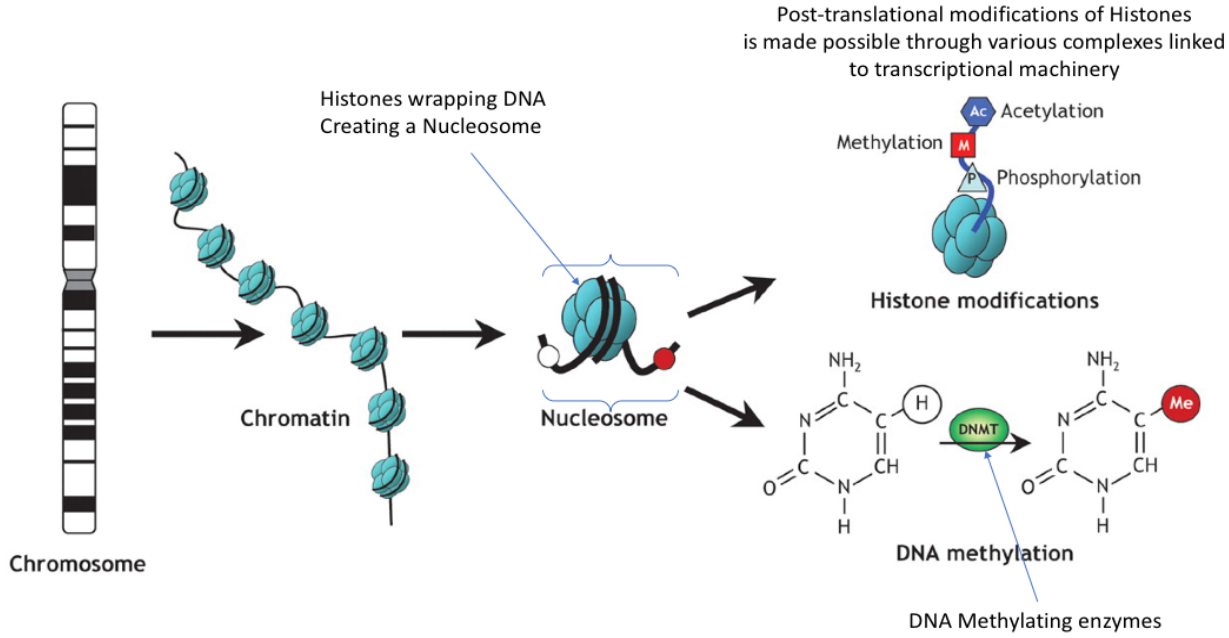


Figure 1

Illustration showing the basic organisation of DNA and its relation to histones. Chromatin is made up of discrete structures called nucleosomes. A nucleosome is essentially DNA around 4 histone molecules. As depicted here each aspect of this is under tight regulation with the help of covalent modifications directly being made to the very molecules themselves. Adapted and modified under **Creative Commons Attribution**

License 3.0 from Professor Le Dinh Luong, Genetics. OpenStax CNX. Jul 29, 2009 http://cnx.org/contents/cbba1ce8-b158-45af-a907-b99e32dff7be@1.1.

A well-known chemical modification of DNA is the methylation of the Cytosine residues. This modification isn't just present in a random haphazard manner but rather when determining which Cytosine residues in DNA are targets for methylation, greater than 90% of methylated Cytosines are found in the dinucleotide pattern also known as a 'CpG' pattern (Bird, 1986). Keeping things simple one can conclude from what we presently know is that DNA methylation status of a promoter region is connected with the gene's transcriptional activity. To generalize methylation is a repressive mark.

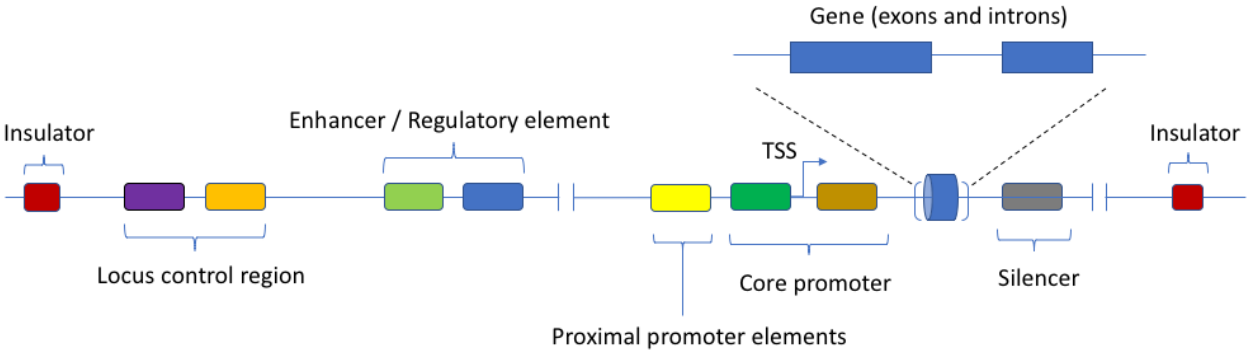


Figure 2

A functional level depiction of the basic structure of a gene and its transcriptional control modules based on Levine and Tjian (2003). DNA sequences shown here are an idealised example of a Pol II dependent gene. The 'cis-regulatory' elements shown here are the focus of this dissertation. TSS: Transcription start site.

The other essential chapter of epigenetics includes the protein scaffold of DNA, the histones. The very discovery of histones and its modifications led to the hypothesis of the 'histone code' (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Which essentially proposed that the transcription of genetic information encoded in DNA is in part regulated by chemical modifications to histone proteins, primarily on their unstructured ends. There are a few major types of histone modifications have been rather well characterised. These include methylation, acetylation, ubiquitination and phosphorylation (Fig. 1) (Strahl and Allis, 2000).

At a functional level, metazoan genes contain highly structured regulatory DNAs that direct complex patterns of expression in many different cell types during development (Fig 2). A typical eukaryotic gene as shown in Fig. 2 is likely to contain several enhancers that can be located in 5′ and 3′ regions, as well as within introns. Each enhancer is responsible for a subset of the total gene expression pattern; they usually mediate expression within a specific tissue or cell type. A typical enhancer is around 500 bp in length and contains several binding sites called 'cis-regulatory' elements for different sequence-specific protein/transcription factors (discussed below) (Davidson, 2001). The core promoter is usually composed of ~60 bp at the transcription start site (Smale and Kadonaga, 2003). This elaborate organisation of the regulatory DNA sequence

permits the detailed control of gene expression. Indeed, a defining feature of eukaryotic gene regulation is the use of multiple enhancers, silencers and promoters to control the activities of a single transcription unit with the help of several protein complexes as discussed below.

Transcription factors and their functional relation to chromatin

Differential gene expression is essential to the very concept of multicellularity in eukaryotic organisms. Genes need to be turned activated and repressed with high spatiotemporal specificity in order to drive organogenesis and post-developmental physiology of any multicellular organism (Imamoto, 1973; Levine and Tjian, 2003). Today it is a well-established concept that more 'complex organisms' don't necessarily have more genes, but rather a more complex way of controlling their regulation (Phillips 2008). Eukaryotes rely on a group of proteins known as transcription factors (TFs), which allow them to finely influence the spatiotemporal activity of genes and thus in turn complexity. The yeast genome encodes a total of about 300 transcription factors. In contrast, the genome of *C. elegans* and *Drosophila* encodes at least 1,000 transcription factors in each organism. For humans, the analyses of the complete genome sequence estimated the presence up to 3,000 sequence-specific DNA-binding TFs (Vaquerizas et al., 2009). There are several way of classifying TFs, but they are often classified according to the tertiary structure of their DNA-binding domains (Latchman, 2004). In terms of the basic structure of TFs (Latchman, 1997), one can describe them by domain-based approach, each TF protein should essentially have the following domains:

- DNA-binding domain (DBD) that binds to specific sequences of DNA known as 'cis-regulatory' elements (Fig. 2)
- Trans-activating domain (TAD), which contains attachment sites for other proteins such as co-regulators namely the Mediator Complex
- Signal-sensing domain (SSD) / Ligand binding domain (LBD), this is an optional domain sensing external signals and, in response, transmits these signals to the rest of the transcription complex.

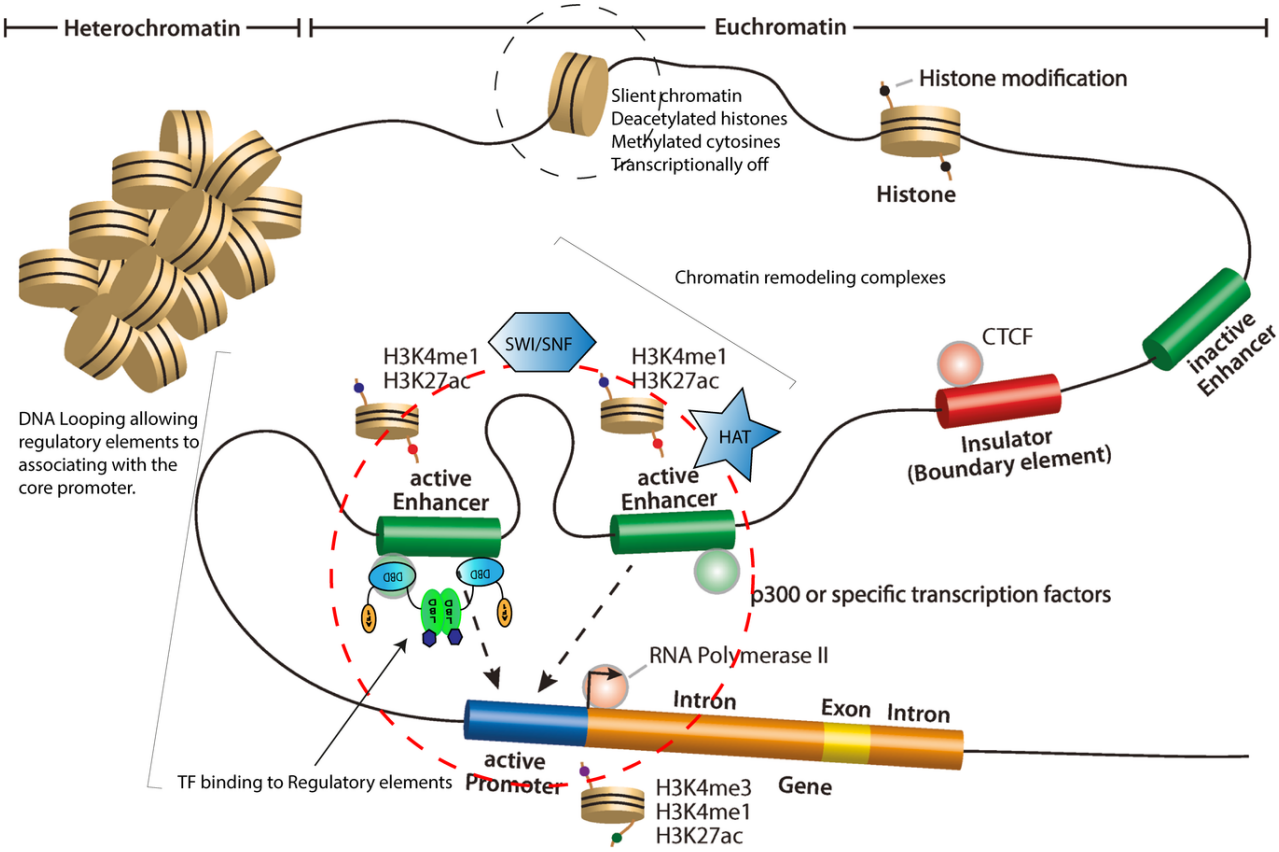


Figure 3

Illustration showing an overview of a functional relationship between cis-regulatory elements, trans-regulatory factors, histone modification and their effect on transcriptional initiation. This figure is essentially a compound view of the process depicted in Fig. 1 and Fig. 2. Red dotted circle: represents the focus of this part of the dissertation. The region shows an intricate mechanism of how TF/NRs are recruited to enhancer elements and set a highly regulated chain of events leading to Pol II-dependent RNA transcript production. HAT and SWI/SNF are histone remodelling complexes associated with the initiation of transcription. Figure adapted and modified with permission from Kratochwil and Meyer, (2014).

Nuclear Receptors as a superclass of transcription factors

Nuclear receptors (NRs) belong to one of the most abundant classes of transcription factors found in metazoans. They serve a wide variety of functions involved in gene expression, such as development, reproduction, metabolism, inflammation and toxicology (Laudet and Gronemeyer, 2002; Laudet and Gronemeyer, 2002a). Many NRs function as ligand-activated transcription factors thus providing a direct link between signalling molecules (either extra- or intra-cellular) and transcriptional responses. Thanks to computational bioinformatics many NRs have been discovered in various organisms. Surprisingly some so-called 'simpler organisms' like *C. elegans* have a far larger repertoire of NRs encoded in their genome compared to humans. NRs in *C. elegans* underwent an explosive expansion and divergence, which resulted in astounding 284 receptors, compared to 48 for humans and 21 for flies (Maglich et al., 2001; Robinson-Rechavi et al., 2005; Taubert et al., 2011). In an evolutionary context, direct access to the control centre (DNA

& Nucleus) provides a functional and practical edge over an extensive signalling cascade based expressional regulation, which in a sense eases evolution (Babonis and Martindale, 2017), this hypothesis explains the vast expansion of NRs in nematodes (Taubert et al., 2011).

Many NRs identified have no known natural ligand, and are hence referred to as 'nuclear orphan receptors' (Robinson-Rechavi, 2003). Despite many advances in the field of molecular biology for many NRs, in particular, orphan NRs, the mechanism of transcriptional activation, recruitment of coactivators, and agonist-mediated activation remains obscure. Lack of discovery of many ligands for orphan receptor does not necessarily mean that all documented orphan NRs will remain orphan but rather hints towards the challenge in finding them. However, a possibility does exist that many NRs may function in a ligand-independent fashion, this may be an impossible fact to prove. Thus, this issue of whether all NRs have endogenous ligands is still a debated (Schupp and Lazar, 2010).

As a general proof of concept, I outline the mechanism of action of well-characterised ligand-dependent and some orphan NRs. As previously stated NRs belong primarily to transcription factor superclass, and like other transcription factors, they contain a distinct DNA binding domain that allows for target sequence recognition, a transactivation domain that possesses the ability to activate transcription through either interaction with cofactors or post-translational modifications. And they also contain a ligand binding domain, whose name is self-explanatory of its function (Fig. 4).

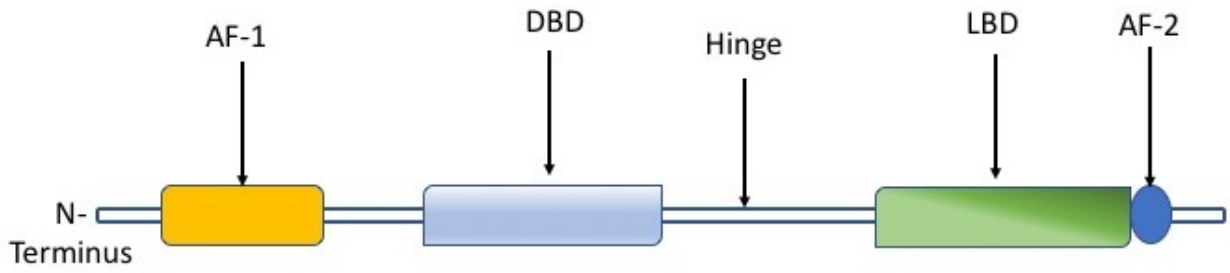


Figure 4

Basic structural anatomy of NR protein sequence. Nuclear receptors are composed of independent functional domains that include the DBD and LBD, the primary functions of which are to recognize specific DNA sequences and ligands, respectively. Nuclear receptors generally possess two transcription activation functions/domains (AF-1 and -2) located at the N and C termini. Adapted form Giguère, (1999).

Many research groups have focused on every aspect of the NR anatomy, of my interest is the (trans)activation domain. Many NRs have at least two activation domains, the ligand-independent activation function (AF)-1, which generally resides in the N-terminal region, and the ligand-dependent AF-2, which is localised in the C-terminal ligand-binding domain (LBD). Full transcriptional activity of an NR is achieved through a synergism between both of its AFs. Moreover, the transcriptional potential of each activation function is dependent on extrinsic

determinants such as the gene promoter, binding partners, cofactors, cell type, and post-translational modifications. All AF-1/N-terminal regions of NRs studied so far are the least conserved region among NRs, both in size and sequence, for example, the N-terminal domains of the vitamin D receptor and the mineralocorticoid receptor are 23 and 602 amino acid residues long, respectively (Krust et al., 1986; Segraves, 1991). Consequently, the activation ability of AF-1 domains has been shown to vary considerably between different NRs. This is in contrast to the AF-2 domain, localised within the LBD, which is much more conserved. In fact, the AF-2 domain consists of a surface created by different structural elements in the LBD (Wärnmark et al., 2003).

The textbook model of nuclear receptor action is often represented by an inactive cytoplasmic receptor in a complex with cytoplasmic chaperones that, upon ligand binding, translocates to the nucleus and activates gene expression (Fig. 4). This model holds true for many steroid receptors (Pratt, 1997), many other NRs are constitutively nuclear and often bound to DNA response elements in the absence of their ligand. It is also now widely accepted that in the absence of ligands, many nuclear receptors can act as a strong repressor of gene transcription (Hörlein et al., 1995; Baniahmad et al., 1992; Chen and Evans, 1995; Damm et al., 1989; Sap et al., 1989; Graupner et al., 1989). Anatomy paves the way for function when it comes to the mechanism of action for NRs. A critical aspect of an NR's function is its ability to specifically bind DNA regulatory elements to exert its function as a transcriptional initiator and controller. To modulate the transcription of their target genes, nuclear receptors interact with co-regulatory proteins. Nuclear receptors have been shown to associate with various components of the general transcription machinery, co-repressors, co-activators, and the co-integrator CBP (CREB-binding protein)/p300. Co-repressor proteins may function by recruiting histone deacetylases, thus producing a repressive epigenetic mark (Biddie and Hager, 2009; Hager et al., 2009; Misteli, 2001; Cohen et al., 2000). Upon ligand binding, the repressor complex dissociates from the receptor, which is then free to interact with the co-activator complex (Perissi et al., 2004; le Maire et al., 2010). The receptor/co-activator complex may contain more than co-activators, including regulatory non-coding RNA molecules referred to as SRA, p/CAF (p 300/CBP-associated factor), CBP/p300, and other components (Lanz et al., 1999). SRC-1, p/CAF, and CBP have been shown to possess intrinsic histone acetylase activity leading to a de-repression of the chromatin structure (Kim et al., 2001).

The molecular mechanisms through which nuclear receptors recruit the aforementioned co-regulator complexes (Fig. 5) are relatively well understood by Watson et al., (2012a). The NRs utilises its AF domains to carry out the recruitment. Experiments have identified critical sequence motifs in co-regulator proteins that mediate ligand-dependent interactions with nuclear receptors, the ligand-dependent recruitment of co-regulatory molecules is a feature of the AF-2 domain in particular. Many of the co-activator complexes recruited to nuclear receptors do so through a conserved motif with the sequence LxxLL (Heery et al., 1997). An analogous sequence motif (LxxH/IIxxxI/L) can be found in co-repressor proteins (Nagy et al., 1999; Hu and Lazar, 1999). Crystal structure analysis has demonstrated that NR and co-repressor proteins adopt a conformational

change that allows them to bind in the same hydrophobic groove on LBDs and therefore recruitment of a co-activator and a co-repressor becomes mutually exclusive (Nolte et al., 1998; Xu et al., 2002; Wang et al., 2006; Phelan et al., 2010). As discussed above, the class of co-regulator that is recruited to the LBD depends upon the ligand-dependent positioning of the C-terminal helix (AF-2) of the receptor. The lack of size and sequence conservation in the AF-1 domain has made studying the domain challenging thus not much data is available regarding AF-1 sequence motifs. This lack of sequence conservation is in fact partially contributed by the presence of intrinsically discorded regions (IDRs), and AF-1 domains belong to a large category of intrinsically disordered activation domains. This property of intrinsic disorder is considered to be key to the function of AF-1 domains (Hilser and Thompson, 2011). Several different co-activators/Mediator subunits, which also have IDFs, have been shown to bind to the AF-1 domain (discussed later) (Hilser and Thompson, 2011; Warfield et al., 2014). For instance, the TATA box-binding protein (TBP), the cAMP response element binding protein-binding protein, the vitamin D receptor-interacting protein 150 and the Alteration/deficiency of activation 2 protein (Ada2p) are known to bind glucocorticoid receptors' (GRs) AF-1 domain (Ford et al., 1997; Henriksson et al., 1997; Almlöf et al., 1998; Hittelman, 1999). Furthermore, in contrast to AF-2, the AF-1 domains contain many post-transcriptional modifications (PTM) such as phosphorylation, acetylation and sumoylation, which have been shown to affect the transcriptional activity of NRs (Weigel, 1996; Anbalagan et al., 2012).

In order to further understand the function of NRs, we first need to look at how these TFs are classified. There are several ways of classifying NRs, a unified system of NR nomenclature was proposed in 1999, the NR superfamily is divided into 7 subfamilies in the Nuclear Receptors Nomenclature (1999) and is based on sequence homology suggested by Laudet, (1997) of the evolutionary conserved DBDs, as well as LBDs that are consistent with the evolutionary scheme based upon a large number of sequenced genomes (King-Jones and Thummel, 2005; Reitzel and Tarrant, 2009; Srivastava et al., 2008; Bridgham et al., 2010). Most NRs bind to DNA as dimers, either as homodimers or heterodimers, some, however, bind to DNA as monomers. Therefore, some have used this very dimerization ability of NRs to classify them. This classification essentially divides the NR superfamily into four classes (Mangelsdorf et al., 1995). Class I receptors are recognized by their ability to bind as homodimers to inverted DNA repeats, while Class II receptors heterodimerize with the retinoic X receptor (RXR), binding to direct repeats (with exceptions). Class III and IV receptors bind as homodimers to direct repeats and extended core sites as monomers, respectively.

Examples of Class I receptors include the glucocorticoid receptors (GRs), estrogen receptors (ERs), progesterone receptors (PRs), mineralocorticoid receptors (MRs) and androgen receptors (ARs), which, when unliganded, are complexed to chaperones (e.g. Hsp70, Hsp90) in the cytosol and dissociate into the nucleus to affect gene expression after ligand-induced allosteric change has occurred (Beato, 2000). NR action is then executed depending on the set of associated

proteins (Hermanson et al., 2002; Kraus and Wong, 2002), such as co-activators (Cosma, 2002) and co-repressors (Privalsky, 2004), as well as chromatin structure by managing access of the transcriptional machinery to specific gene sequences (Khorasanizadeh, 2004).

Class II receptors bind as heterodimer complexes and are also located within the nucleus complexed with co-repressors in a repressive cognate state, thus keeping transcription off. After a ligand binding induces a conformational change, histone acetylase complexes are recruited, reversing the repressive effects and assembly of the basic transcriptional machinery, this will be discussed later in detail. The repression is maintained by NCoR and SMRT co-repressor complexes (Chen and Evans, 1995; Hörlein et al., 1995) associated with histone deacetylases (Watson et al., 2012). Cell-autonomous feedback with several NRs in Class II is observed because they bind ligands produced in the same cell (Sever and Glass, 2013). This class of receptors includes e.g. retinoic X receptor (RXR), thyroid hormone receptor (TR) and vitamin D receptor (VDR).

Class III NRs function probably similarly as Class I NRs, with homo-dimers binding commonly to direct repeats (e.g. HNF4, COUP-TF, RXR) (Rastinejad et al., 2015). Class IV NRs compromise a group of NR monomers binding to single DNA half-sites (e.g. ROR, NURR family) (Rastinejad et al., 2015; Mangelsdorf et al., 1995).

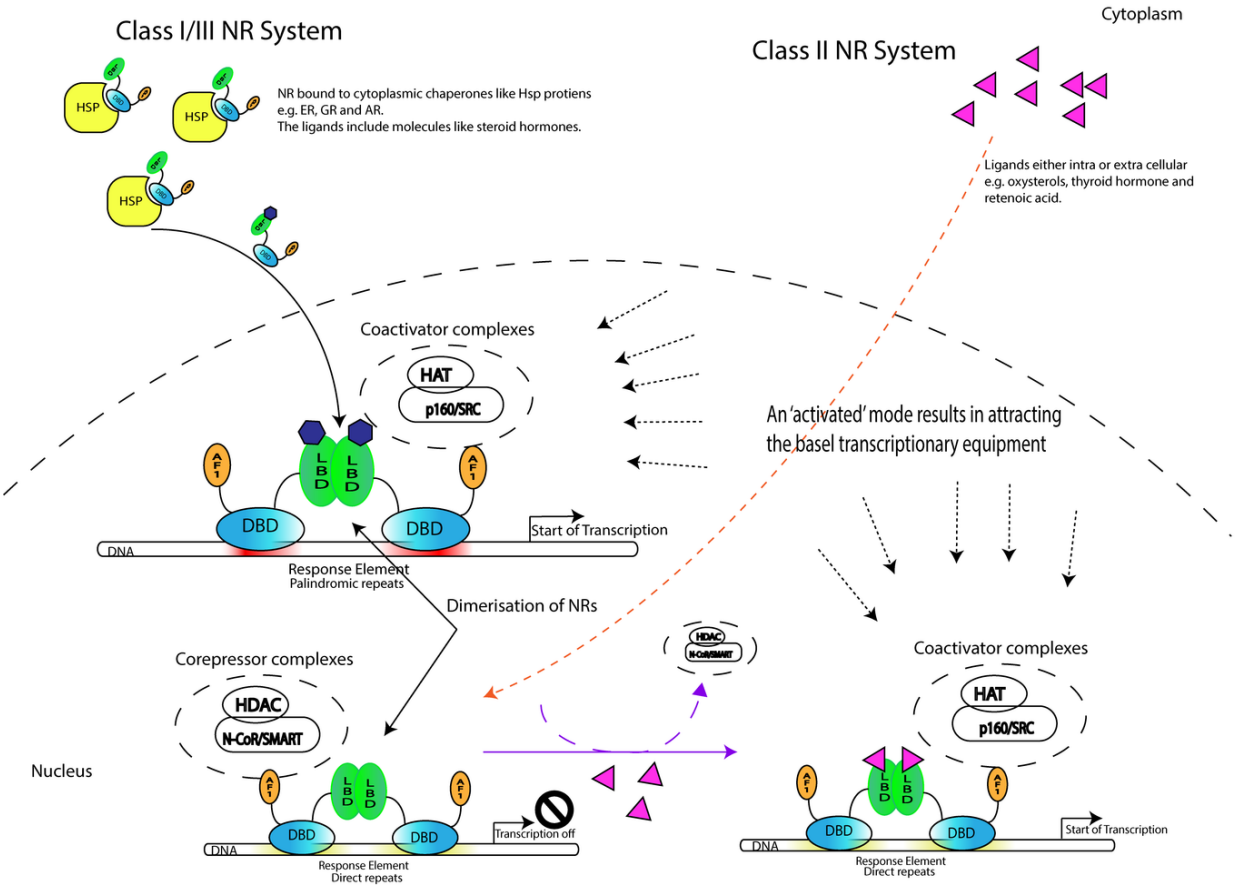


Figure 5

Basic functional outline of the mechanism of action of different classes of NRs. Three different classes of NRs are demonstrated here. Class I and III NRs include receptors like AR, GR, ER, MR and HNF4alpha, COUP respectively, there are anchored in the cytoplasm by chaperone proteins like Hsp70 and Hsp90. Class II NRs include thyroid hormone receptor and the retinoic X receptor, in contrast, these receptors reside in the nucleus bound to their specific DNA response elements even in the absence of ligand. As shown here NRs can be modulated by ligands that either activate or repress gene targets. Repression is mediated by complexes that have corepressors (SMRT/N-CoR and histone deacetylases or HDACs), among other components. Activation requires the coactivator complexes (p160 family members and histone acetyltransferases or HATs). The repressive and activating complexes block or promote transcription. Furthermore, receptors can be organised into distinct oligomeric states such as heterodimers with the common partner RXR, homo-dimers, or monomers. The non-steroid receptor heterodimers and many homo-dimers bind to direct repeat response elements with various inter-half-site spacings. Steroid receptor homodimers mainly use palindromic DNA elements, where the two half-sites are in an inverted repeat fashion. Other receptors (not shown here) use monomeric sites extended at their 5′ end with short sequences used for selectivity. The illustration was adopted from Rastinejad et al., (2013); Sever and Glass, (2013).

Taken together, transcriptional regulation by nuclear receptors incorporate several major players involved in epigenetic modifications, hence understanding the chromatin status and histone code is vital to understand the big picture. To summarise, there are three chromatin states in relation to NRs, first a normal chromatin in the absence of receptor that displays a basal level of histone acetylation and transcription; second a repressive chromatin state with deacetylated histones and no transcription in the presence of the unliganded receptor; and lastly an active chromatin with high levels of histone acetylation and transcription in the presence of liganded receptor.

Transcriptional initiation and the Mediator complex

Having an overlook at how NRs fit into the complex world of gene regulation I now look at the next steps required for transcriptional induction. Once bound to DNA and after the initial phase of local chromatin remodelling i.e., modifying the chromatin from repressive to an active state, NRs further start the process of recruiting general transcription factors and other co-activators namely the mediator complex, which then stabilize the preinitiation complex (PIC) containing the RNA polymerase II. First, we have to understand what the Mediator complex is before trying to dismantle its function. The role that mediator plays in the initiation of transcription is somewhat complicated and not all the aspects have been understood to date.

The Mediator complex, as the name implies, is a multi-subunit complex consisting of 25 to 30 subunits (yeast and human, respectively) with an overall molecular weight of >1MDa (Bourbon, 2008; Thompson et al., 1993). It was first discovered in yeast as a transcriptional cofactor (Kelleher et al., 1990; Flanagan et al., 1991; Kim et al., 1994), in the past many names have been given to the mammalian version of the Mediator complex in literature, such as TRAP (thyroid hormone receptor-associated protein) (Fondell et al., 1996), ARC (activator-recruited cofactor) (Näär et al.,

1999), or DRIP (vitamin D receptor-interacting protein) (Rachez et al., 1998). The Mediator complex has been biochemically isolated in at least two forms: the small mediator and the large mediator. Structurally as a whole one can think of the complex as four modules; the 'head', 'middle', 'tail' and the 'CDK8 kinase' module. The way in which these modules associate is a matter of debate, but from the present experimental data, one can divide the complex into either, the smaller, core complex (600 kDa) and the larger mediator (1.2 MDa) (Taatjes, 2002). The head, middle and tail modules form a relatively stable 'core' structure, the kinase module — which consists of four subunits (cyclin-dependent kinase 8, MED12, and MED13, but lacks the MED26 subunit) associates reversibly with the Mediator (Cevher et al., 2014; Plaschka et al., 2015). The CDK8 kinase module behaves in a context-specific manner to either repress or activate transcription, depending on the transcription factors and/or target gene promoters (Nemet et al., 2014). In spite of the structural complexity, the underlying modular architecture, in essence, reflects a division of labour at the functional level.

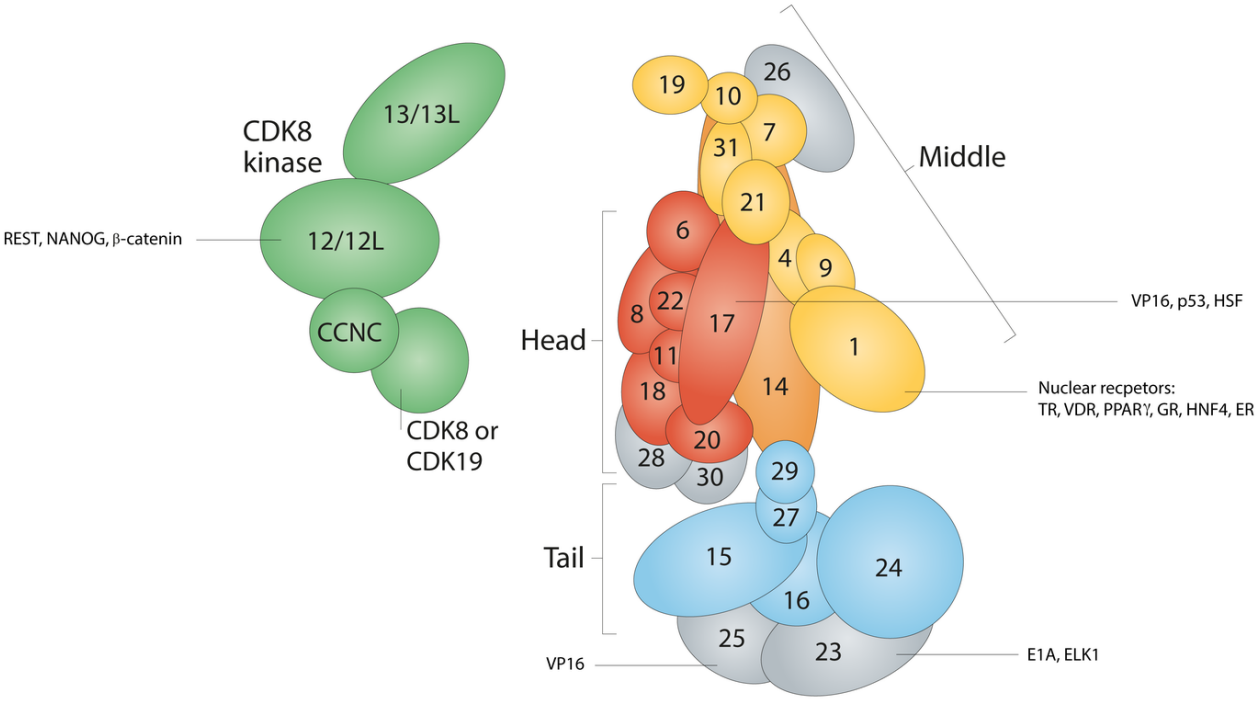


Figure 6

This illustration is an attempt to depict, from published data (Guglielmi, 2004; Belakavadi et al., 2008; Baumli et al., 2005; Larivière et al., 2006; Tsai et al., 2014; Robinson et al., 2015). The four modules: the head module (in red), the middle module (in yellow) and tail module (in blue), which function as the main 'core' complex and the CDK8 kinase module (in green), which is transiently associated with the complex as shown in Fig 5. Mediator subunit 14 (MED14), which links all three main modules (head, middle and tail), is indicated in orange. The exact localization of five metazoan-specific subunits (MED23, MED25, MED26, MED28 and MED30) with the whole complex remains to be designated. These subunits have been shown here in grey according to a proposal discussed by Malik, (2016). Furthermore, I try to show documented subunit:NR and subunit:protein interaction partners. MED23's interaction with and an adenovirus protein EA1 and a RAS-

MAPK-dependent factor ELK1 was shown by Boyer et al., (1999); Wang et al., (2005). MED1's targeting by ligand-inducible NRs have been very well documented (Rachez et al., 1999; Ge et al., 2002; Ge et al., 2007; Malik et al., 2002; Hittelman, 1999; Kang et al., 2002; Malik et al., 2004; Ito et al., 2000). Developmental pathways also have been shown to converge at the Mediator converge on the Mediator proteins MED12 and MED13 a few examples include Wnt/β-catenin signalling in *D. melanogaster* (Carrera et al., 2008; Kim et al., 2006a) and a negatively acting neuronal developmental transcription factor REST in *Danio rerio* (zebrafish) (Wang et al., 2006a). Last but not least an interaction of the embryonic stem cell transcription factor NANOG has also been observed (Tutter et al., 2008). This illustration was adopted with permission from Soutourina, (2017).

As depicted in the figure above, the Mediator complex has the ability to bind various transcription factors and many other proteins and integrate these signals to the basal transcriptional machinery (Malik and Roeder, 2010; Taatjes, 2010; Conaway and Conaway, 2011; Allen and Taatjes, 2015). Furthermore, it also functions as the initiator of the general transcription factor assembly i.e. aids in the formation of the transcription preinitiation complex with Pol II. It is tightly associated with the general transcriptional machinery by making contact to RNA polymerase II and interacting with transcription factors and is thus by many regarded as a general transcription factor (Takagi and Kornberg, 2005). The general transcription factors (GTFs) include TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Holstege et al., 1998; Soutourina et al., 2011; Thompson and Young, 1995). Formation of the transcription preinitiation complex is nucleated by the TATA-binding protein (TBP) at the core promoter site, a subunit of TFIID that binds the TATA element and induces DNA looping i.e. enhancer and basal promoter proximation, this has been shown to be a MED1 dependent process (Poss et al., 2013). In the absence of a TATA sequence, PIC assembly is initiated by the association of other subunits of TFIID with other promoter elements (Pugh and Tjian, 1991). The next steps essentially involve the loading of the other GTFs on to the core promoter, the process of GTF loading is in fact also shown to be Mediator guided (Kornberg, 2007; Poss et al., 2013). Fig. 7 illustrates a visual summary of the steps involved in Mediator based PIC formation.

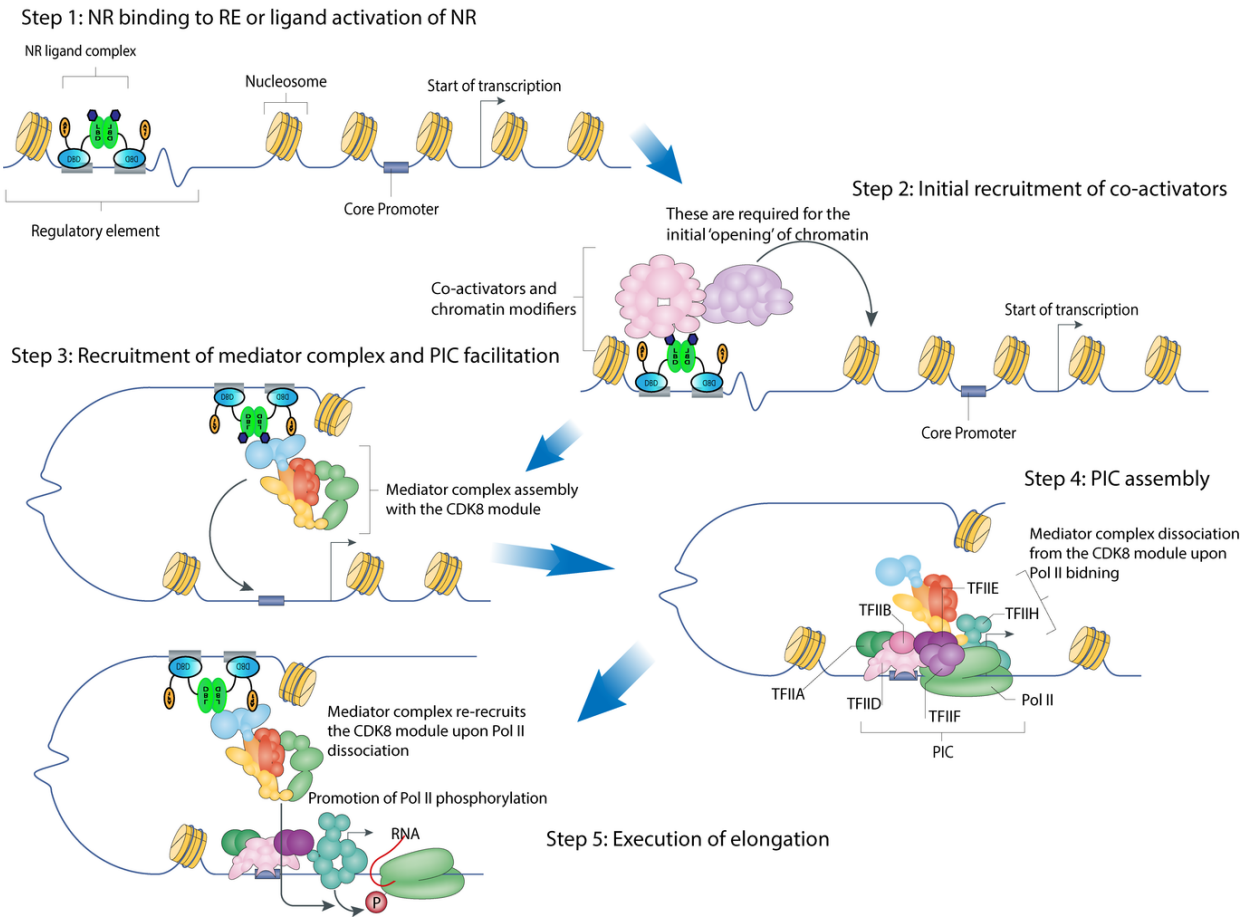


Figure 7

This illustration shows the current model of Mediator assisted PIC formation and transcriptional initiation. Step one shows the idealised pathway with the attachment of a liganded NR bound an enhancer/regulatory element upstream of a transcriptional start site. At this point, the chromatin is in a repressive state. Step 2 shows the 'opening' of the chromatin structure as induced by the recruitment of co-activators as previously demonstrated in Fig .2. The resulting chromatin is characterised by distinct covalent modifications. Step 3. show the activator-mediated recruitment of the Mediator complex with the CDK8 module. Step 4. is the PIC assembly, entailing the GTFs (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and Pol II (Eychenne et al., 2016; Esnault et al., 2008; Johnson, 2002; Larivière et al., 2006), followed by the initiation of transcription right after the displacement of the CDK8 module with Pol II. After the release of Pol II the CDK8 module reassociates with the Mediator and in the likely involved in the elongation phase of transcription (Takahashi et al., 2011). This illustration was adopted with permission from Soutourina, (2017).

From the information discussed above, it should be clear that the Mediator is not only the basis for regulated transcription, but it is absolutely required for almost all transcription of nearly all Pol II-transcribed promoters, in a way it is no less important for transcription than Pol II itself (Eyboulet et al., 2015). Not only is the Mediator important for positive regulation but also for negative regulation of transcription (Knuesel et al., 2009). Many commonly refer the Mediator as a co-activator, it is rather a co-activator, a co-repressor, and a general transcription factor all dependent upon cellular context (Elmlund et al., 2006). The Mediator may be seen as a signal processor. It

transduces regulatory information from enhancers to promoters in the entire evolutionary line-up of species from yeast to humans.

Other functions of the Mediator indeed go far beyond the focus of my dissertation. However, some other noteworthy functions include the elongation of the Pol II transcript (Conaway and Conaway, 2013), chromatin looping (Park et al., 2005) and histone/chromatin modification (Meyer et al., 2008). This far-reaching potential of the Mediator makes it evident that the extensive and complex regulatory ability of the mediator complex, of which much is yet to be discovered. To further complicate matters the possibility of post-translational modifications, on the Mediator's subunits, adds another layer of regulation to control gene expression by altering subunit activity in countless ways (Nagalingam et al., 2012).

Mediator at the evolutionarily conserved nexus of cellular signalling

At this point, it has become more than clear that the Mediator not only functions as a site-specific/cell-specific manifold for PIC assembly but is also situation-specific transcriptional responder i.e. cellular cues, either intra/extracellular converge at this nexus as already pointed out in Fig 6. We have already touched on the fact that NRs may have an evolutionary advantage when it comes to having a direct link to DNA as stimuli and acute biological responses can be coupled without the need for an extensive signalling cascades, which eases evolution (Babonis and Martindale, 2017), a hypothesis that may support the huge expansion of NRs in nematodes (Taubert et al., 2011). An efficient way of classifying proteins across species is largely based on comparing the most conserved regions of the proteins. The Nuclear Receptors Nomenclature, (1999) uses sequence homology of the evolutionary conserved DBDs, as well as LBDs that are consistent with the evolutionary scheme based upon a large number of sequenced genomes. When viewing the Mediator subunits under the evolutionary 'microscope' it is somewhat more challenging.

In evolution, Mediator emerged in eukaryotes, coinciding with the emergence of other general TFs (Takagi and Kornberg, 2005), and is conserved from unicellular eukaryotes to metazoans (Bourbon, 2008). However, throughout eukaryotic evolution, the primary sequences, the subunit composition, and function of Mediator diversified but structural homologues of all Mediator subunits are present in yeast and humans (Poss et al., 2013; Bourbon, 2008). Poor sequence conservation between yeast and human Mediator is observed on the basis of multiple sequence alignments and secondary structure features, although a set of conserved Mediator subunits have been identified in most eukaryotes. Lack of high sequence homology in the subunits is due to the similar phenomenon seen in the AF1 domains of NRs (Wärnmark et al., 2003). In most metazoan, plant and fungi Mediator subunits exist intrinsically disordered regions (IDRs) (Tóth-Petróczy et al., 2009), their presence might partially explain the divergence of Mediators' primary sequences as these generally tend to diverge more rapidly over the course of time (van der Lee et al., 2014). However, it is relevant to point out that even though the sequence conservation is

relatively weak for some subunits, the location of IDRs within the primary is similar, suggesting general structural conservation (Nagulapalli et al., 2015). This structural conservation is what plays a vital role in Mediator's adaptability, both conformational and regulatory, and its capacity for multiple protein-protein interactions (Tóth-Petróczy et al., 2009). In part one can also explain the Mediator's divergence by the increasing diversity of its binding partners like DNA-binding TFs/NRs. The subunit composition depends on the organism, and the overall number of Mediator subunits increased during eukaryotic evolution. Although many of the basic functions of Mediators are conserved from yeast to human (TF binding and Pol II recruitment) some of the more advanced capabilities like binding to various chromatin/Pol II-associated factors is not possible in yeast as they are simply not found in the yeast genome (Jishage et al., 2012; Hu et al., 2006a; Lai et al., 2013; Kuuluvainen et al., 2014).

Specialised roles of Mediator subunits in the context of cellular signal processing

Defining each of many documented roles of the Mediator are far beyond complex for the purposes of my dissertation. The aim here is to show the *'transducible'* capability of the Mediator or rather it being at an endpoint of evolutionary conserved diverse signalling pathways. As visualised in Fig. 6, some Mediator subunits have shown major interactions with many NRs. Namely, MED1 is a target of ligand-inducible nuclear receptors. MED1 provides a docking surface for nuclear receptors that entails interfacing between a given NR and the transcriptional machinery. In the case of MED1, the AF2 domains of receptors are predominantly responsible for the interactions, which contain the conserved LXXLL sequence motif (Zhu et al., 1997). NRs that have identified as MED1 regulators include the ligand-bound thyroid hormone receptor, vitamin D receptor (Rachez et al., 1999), peroxisome proliferator-activated receptor-γ (PPARγ) (Ge et al., 2002; Ge et al., 2007), hepatocyte nuclear factor 4α (HNF4α) (Malik et al., 2002), glucocorticoid receptor (Hittelman, 1999) and the oestrogen receptor (Kang et al., 2002). Interestingly, for MED1 regulation of PPARγ in cultured mouse embryonic fibroblasts, these LXXLL motifs are not required, which may suggest that MED1 regulation of gene transcription through alternative mechanisms in a context-dependent manner (Ge et al., 2007). Jia et al., (2014) have reported how the functional relationship of MED1 and NRs is interconnected in liver metabolism regeneration and carcinogenesis. Liver function is heavily dependent upon members of the NR family (Wagner et al., 2011), examples include peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR), liver X receptor (LXR), constitutive androstane receptor (CAR), farnesoid X receptor (FXR), glucocorticoid receptor (GR), and others. The reason for this high 'NR' dependence may be the fact that the liver has a 'ligand' rich milieu and the liver cells have evolved and adapted using these ligands to regulate energy homeostasis, detoxification of xenobiotics, and the formation and excretion of bile acids. MED15 subunit is another excellent and interesting example of a diversified capability of individual subunits. It was identified as a target of sterol regulatory element-binding

protein 1α (SREBP1α) and is a key effector of SREBP-dependent gene regulation and control of lipid homeostasis in Metazoans (Yang et al., 2006). Furthermore, as reported by Taubert et al., (2006) that MED15 in *C. elegans* coordinates expression of metabolic genes involved in lipid metabolism by interacting with NHR-49, which is one of the major controllers of fasting response and β-oxidation genes. At the same time, MED15 has also been implicated as a target subunit for the conserved transforming growth factor-β (TGFβ)/Nodal/Activin/Smad2 developmental signalling pathway (Kato et al., 2002). Perhaps the fascinating role of MED15 hasn't even been thoroughly characterised. In an exhaustive immunoprecipitation-mass spectrometry study performed by Malovannaya et al., (2011) showed that the interaction network of MED15 was distinct relative to other Mediator subunits, suggesting it may function independently of Mediator. The authors suggested that the protein is found in two exclusive complexes: the known Mediator complex, but also interacts separately with an E3-ligase TRIM11, which sequesters MED15 subunit away from the main body of the Mediator complex in a relatively stable and discrete complex. Up until now, most, if not all, examples of Mediator subunits that I have illustrated here are showing these subunits as signal receivers or rather transducers, of other established signalling systems like NR, and their role in PIC assembly. However, MED12 subunit might function independently as a regulator of TGFβ signalling in the cytoplasm where it negatively regulates TGF-βR2 through physical interaction (Huang et al., 2012). Keeping that in mind a *C. elegans* RNAi screen identified MED12 as a highly connected 'hub' gene that regulated numerous signalling pathways (Lehner et al., 2006), and others have linked MED12 to WNT–β-catenin signalling pathways and Hox gene expression (Kim et al., 2006a; Moghal and Sternberg, 2003; Yoda et al., 2005). These roles point to a traditional role as a member of the Mediator complex, i.e. acting as an end-point of signalling cascades. The last subunit that I would like to divert most of my attention to and is the focus of this dissertation is the subunit 28. MED28 subunit was shown to be involved in regulating smooth muscle (SMC) development. RNAi of MED28 in NIH3T3 and myoblast C2C12 cell lines lead to up-regulation of smooth muscle genes, whereas over-expression of MED28 repressed the expression of these genes. This data also showed for the very first time that MED28 functions as a negative regulator of SMC differentiation in concert with other Mediator subunits including MED6, MED8, and MED18 within the Mediator head module (Beyer et al., 2007). *MED28* as a gene sequence had already been previously identified as an endothelial cell gene and was named endothelial-derived gene EG-1, its expression was associated with a stimulated state in endothelial and epithelial cells, and it was proposed to play a role in tumour angiogenesis (Liu et al., 2002). Later research, identified it as a protein that interacts with the cytoskeletal protein merlin/Neurofibromin 2, it localises beneath the plasma membrane, it interacts with the actin cytoskeleton and also binds the well-characterised adapter protein Grb2 (Wiederhold et al., 2004). Other studies have found that MED28 also interacts with the Src family members (c-Src and Lck) and the binding results in the activation of c-Src without causing an over-expression of the c-Src (Lu et al., 2006) secondly Lck (Src family kinase) can phosphorylate MED28 (Lee et al., 2006). Huang et al., 2012a provides evidence for MED28 transcriptionally up-regulating genes involved in cell migration namely MEK-

1 and MM2 in breast cancer. Based on the available literature regarding MED28 one can only say that much further analysis in need to understand this multifaceted protein. During the development of our publications (Chughtai et al., 2015; Kostrouchová et al., 2017) a comprehensive review of the Mediator complex, its subunits and predicted functions in *C. elegans* were published by Grants et al., (2015). As our model organism of choice was *C. elegans,* a tabulated list of Mediator subunits in *C. elegans* and a predicted structural model from the comprehensive review has been added here.

Table 1 was based on the comprehensive review of the Mediator complex in *C. elegans* by Grants et al., (2015) (adapted with permission). MED28's sequence number has been corrected based on the evidence provided by my colleagues and myself in the two publications (Chughtai et al., 2015; Kostrouchová et al., 2017).

Table 1: Listing the 29 putative *C. elegans* Mediator subunits, their mammalian orthologues and sequence number. ✯ *W01A8.1* gene is now annotated as plin-1. ✢*F28F8.5* gene encodes MDT-28 the true orthologue of mammalian MED28.

C. elegans subunit	Mammalian orthologue	Sequence number
MDT-1.1	MED1	Y71F9B.10
MDT-1.2	MED1L	T23C6.1
MDT-4	MED4	ZK546.13
MDT-6	MED6	Y57E12AL.5
MED-7	MED7	Y54E5B.3
MDT-8	MED8	Y62F5A.1
MDT-9	MED9	Y62E10A.11
MDT-10	MED10	T09A5.6
MDT-11	MED11	R144.9
MDT-12	MED12	F47A4.2
MDT-13	MED13	K08F8.6
MDT-14	MED14	C38C10.5
MDT-15	MED15	R12B2.5
	MED16	Absent?

MDT-17	MED17	Y113G7B.18
MDT-18	MED18	C55B7.9
MDT-19	MED19	Y71H2B.6
MDT-20	MED20	Y104H12D.1
MDT-21	MED21	C24H11.9
MDT-22	MED22	ZK970.3
MDT-23	MED23	F39B2.4
MDT-24	MED24	F56H9.5
	MED25	Absent?
MDT-26	MED26	C25H3.6
MDT-27	MED27	T18H9.6
MDT-28	MED28	W01A8.1☆ Corrected F28F8.5✢
MDT-29	MED29	K08E3.8
MDT-30	MED30	F44B9.7
MDT-31	MED31	F32H2.2
CDK-8	CDK8	F39H11.3
CIC-1	CycC	H14E4.5

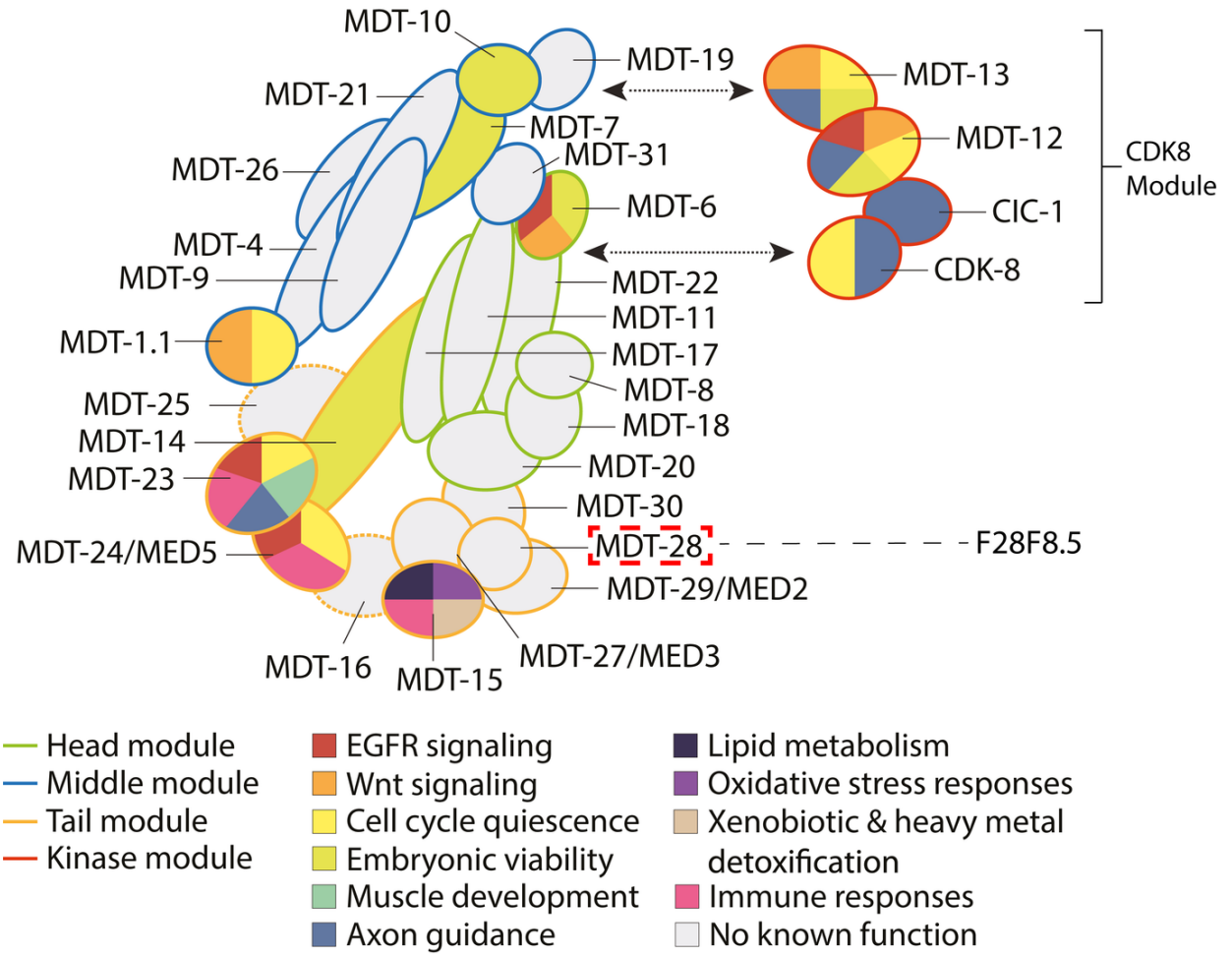


Figure 8

Model showing hypothetical adaptation of *C. elegans* Mediator, based on human Mediator. Subunits labelled with dashed outline lack apparent *C. elegans* orthologues. Additionally, Mediator's involvement in developmental and physiological pathways from known data has been added. Furthermore, the subunit of interest, MDT-28, has been highlighted with thick dashed red outline. Based on our work the true orthologue sequence number has been listed F28F8.5. This illustration was adapted with permission from Grants et al., (2015).

Lipid droplets

For many decades lipid droplets (LDs) had been perceived as inert fat particles, used for storing fat and thus were largely ignored by researchers. The process of lipid storage considered to be relatively benign in which excess fatty acids are converted to neutral lipids and deposited in cytoplasmic inclusions. It was however observed that these fatty cytoplasmic inclusions are ubiquitous found and in most eukaryotic cells and even in prokaryotic cells (Alvarez and Steinbüchel, 2002; Murphy, 1990; Ratledge, 1991; Wältermann and Steinbüchel, 2005). Within and among organisms, LDs physically range greatly in size (diameter < 1–100 μm) this is largely dependent on the cell type e.g. the cytoplasm of adipocytes is essentially an LD, but biochemically

each LD consists of a phospholipid monolayer that surrounds a core of neutral lipids, such as sterol esters or triacylglycerols.

During the last few decades, there has been a growing interest in these intracellular organelles as dynamic regulators of lipid metabolism and their role beyond keeping fat in check. A real interest in LDs began after the discovery of a lipid droplet surrounding protein called Perilipin back in the 1991 (Greenberg et al., 1991). Greenberg and colleagues sparked scientific interest by showing a hormonally regulated major phosphoprotein located in the adipocyte and they coined the term Perilipin. They suggested that Perilipin may provide more than just a structural role due to its ability to respond to acute hormone-induced changes in phosphorylation, including the addition of phosphate by A-kinase and removal of these same phosphates by an insulin-stimulated phosphatase. This discovery was really a milestone in understanding a whole spectrum of metabolic disorders such as metabolic syndrome, insulin resistance and type II diabetes.

Due to a massive recent interest in lipid biology, there have been many speculations into the role of LDs and the associated proteins beyond that of storing fat. These roles are key to understanding not only lipid biology but also the regulation of gene expression, I would discuss this later in detail in the discussion of this thesis.

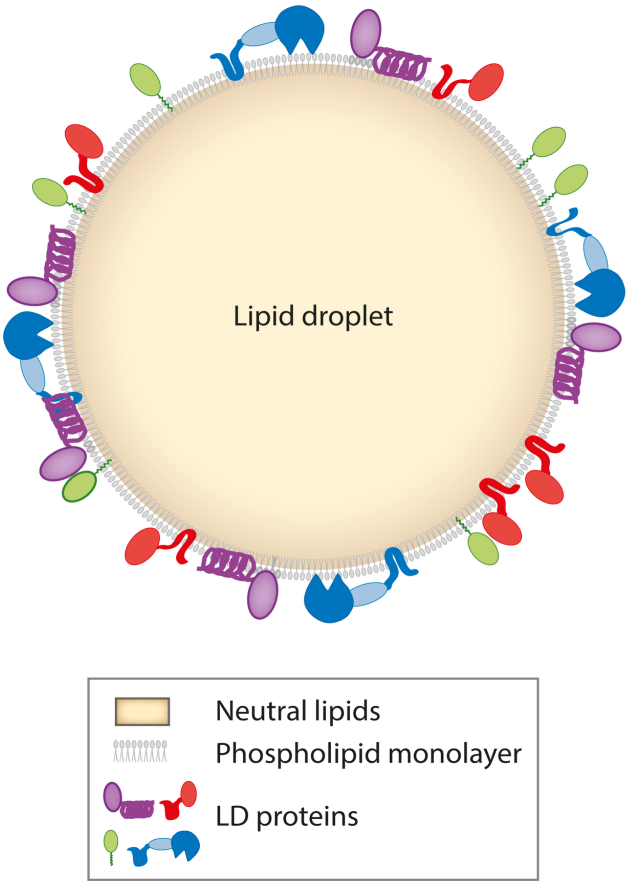


Figure 9

Schematic representation of a cytoplasmic lipid droplet adopted with permission from Walther and Farese, (2012). In this representation, one can clearly see a seemingly simple architecture of the LD which essentially

consists of triglycerides and cholesterol esters surrounded by a phospholipid monolayer. And last but not least a rather complex array of associated proteins, among them Perilipin being the most abundant. As described later in detail these proteins are responsible for regulating the size and metabolic activities of lipidic core. Cellular cytoplasm contains an emulsion of LDs in the cytosol. The LD phase of the emulsion provides a large interface for interactions with amphipathic molecules. The LD surface comprises polar, amphipathic lipids. In mammalian LDs, phosphatidylcholine (PC) is the main surface phospholipid, followed by phosphatidylethanolamine (PE) and phosphatidylinositol (Bartz et al., 2007). Compared with other membranes, LDs are deficient in phosphatidylserine and phosphatidic acid but enriched in lyso-PC and lyso-PE. The primary neutral lipids of the LD core are sterol esters (SEs) and triacylglycerols (TGs). Their relative amount varies between cell types. For example, yeast LDs have a mix of SE and TG, possibly arranged in layers (Czabany et al., 2008). LDs of adipocytes contain primarily TG, and those of macrophage foam cells contain mostly SE. Neutral lipid synthesis is catalysed by various enzymes as discussed later.

Introducing the Perilipin family of proteins

The Perilipin family of proteins have been associated with surfaces of LDs and all carry a conserved 11-mer repeat motif. Previously the Perilipin protein family was known as PAT family of proteins, the name was PAT was derived from names of three proteins, PERILIPIN, ADRP, and TIP47, with each having highly related N-terminal sequences and common affinity for intracellular neutral lipid storage droplets. The protein coat of lipid droplets can vary between droplets within a cell, between metabolic conditions, and between cell types. The current knowledge is based on several proteomic studies (Jolivet et al., 2017; Siegler et al., 2017; Khan et al., 2015; Ding et al., 2012). Furthermore, these studies also identified many other proteins which include proteins involved in lipid metabolism and transport, intracellular trafficking, signalling, chaperone function, RNA metabolism, and cytoskeletal organisation. This heterogeneity of the protein coat is consistent with the dynamic changes in morphology and intracellular location that lipid droplets undergo according to the metabolic state or developmental stage of the cell or organism.

However, the most abundant protein group found on LDs is the Perilipin family. Mammalian genome expresses five unique proteins belonging to this family albeit in a tissue-specific and metabolic state-dependent manner. The table below provides an overview of the Perilipin family members and functional information. In 2010 the unified nomenclature for the mammalian Perilipin-related and PAT-family of intracellular proteins was established (Kimmel et al., 2010).

Table 2: Overview of the Perilipin protein family in mammals. Adopted from Itabe et al., (2017).

Proteins	Alternative name	Major expression sites	Other expression sites	Function
PLIN1	Perilipin A	White adpose tissue	cardiac muscle, brown adipose tissue	hormone-induced lipolysis, large LD stabilization

PLIN2	ADRP, ADFP (human ADRP), Adipophilin	Liver	premature adipocytes, macrophages, sebocytes, mammary gland epithelia, ubiquitously expressed	adipocyte differentiation, small LD generation, LD stabilization
PLIN3	TIP47	ubiquitous	skeletal muscle, neutrophils, mast cells, retinal pigment epithelium, sebocytes	LD stabilization (compensation of PLIN2), PGE2 production, intracellular trafficking
PLIN4	S3-12	White adpose tissue	hMSC (induced during differentiation), skeletal muscle	human adipocyte differentiation
PLIN5	MLDP, OXPAT, LSDP5	cardiac muscle, brown adipose tissue, skeletal muscle	islet β-cells, hepatic stellate cells	LD stabilization, FA supply to mitochondria

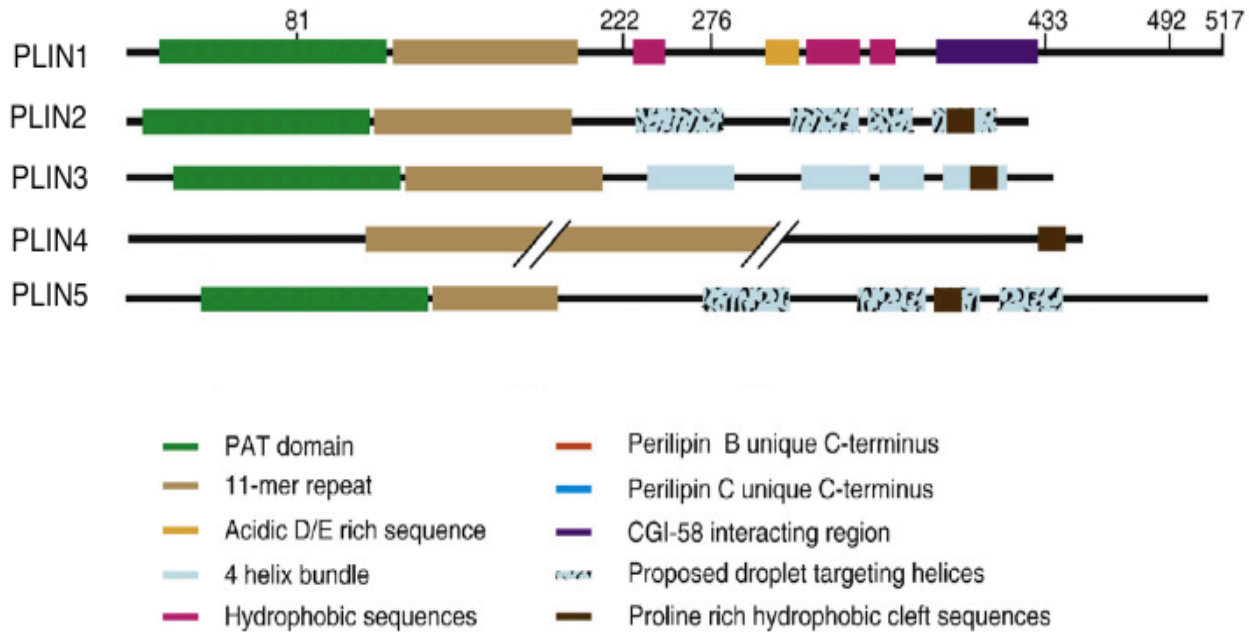


Figure 10

Schematic representation of the PLIN family of proteins showing relevant domains, adopted with permission from Bickel et al., (2009).

To better understand the relevance of the LD protein coat we need to understand the proteins listed in table 2 in a bit more detail. The most well studied member of the family is PLIN1. Most commonly found on adipocyte LDs, which is phosphorylated by cAMP-dependent protein kinase (PKA) during adrenalin-dependent acute lipolysis. Perilipin proteins lack putative transmembrane domains, and it is not completely clear how Perilipins are associated with the surfaces of lipid droplets. Although the PAT domain contributes to the proteins' association with LDs, other determinants of LD surface targeting have been suggested, including various other parts of Perilipin proteins. Several investigations of deletion mutants have showed that N- and C-terminal regions and central portions of PLIN1 and PLIN2 are required for LD targeting (Garcia et al., 2002; Targett-Adams et al., 2003). A recent publication demonstrated that the 11-mer repeat forms amphipathic helices that bind micelles and LDs (Rowe et al., 2016). Accordingly, various point mutations within the 11-mer repeats of PLIN1–3 led to a changed amphipathic amino acid alignment and thus abolished the association with LDs. 11-mer repeats can be found in other proteins, including apolipoproteins and the Parkinson's disease protein α-synuclein (Bussell and Eliezer, 2003), which binds LD-associated proteins in lipid-loaded neurons of the hippocampus (Cole et al., 2001). Moreover, a four-helix bundle structure that resembles that of apolipoprotein E (apoE) was identified in a study of the 3-dimensional structure of the C-terminal region of PLIN3 (Hickenbottom et al., 2004), and similar structures were predicted in homology analyses of PLIN1, PLIN2, and PLIN5 (Rowe et al., 2016; Bickel et al., 2009). PLIN4 has unique structural characteristics, it happens to lack a PAT domain and has a polypeptide length of almost three times the length those of other PLIN proteins. However, like PLIN1, PLIN4 is expressed in adipocytes and it is found on LDs; its 11-mer repeat is likely crucial for LD surface targeting.

Evolutionary aspect of Perilipins

As pointed out earlier prokaryotic and eukaryotic cells contain LDs, which are surrounded by proteins. Yang et al., (2012) tried to show functional conservatism of the LD proteome from bacteria to humans. Furthermore, the work points out that in mammals, the lipid particles found in blood have a similar general structure to intracellular LDs and are surrounded by apolipoproteins (APOs). Yang et al. (2012) suggest that LD proteins have properties in common with APOs. In fact, the apolipoprotein A-V has been found on LDs in hepatoma cells (Shu et al., 2007), apolipoprotein A-I has been found on LDs in white adipose tissue (Kanshin et al., 2009), and APOA-I and APOE have been found on LDs of skeletal muscle cells (Zhang et al., 2011). A very interesting study conducted by Hickenbottom et al., (2004) compared the sequence similarities between Perilipin family and apolipoproteins. They show that the sequence similarities have a clear functional analogy but are distant enough to leave uncertain whether they arose by convergent or divergent evolution. Furthermore, logically thinking it is very unsurprising to discover a close structural relationship between lipid droplet coating proteins and apolipoproteins. The lipidic core of both droplets and lipoprotein particles consists of triglycerides and cholesterol centrally surrounded by a monolayer

of phospholipids (Feingold and Grunfeld, 2000). On a functional level the associated proteins are also analogous i.e. they stabilise assembly, provide docking sites for the appropriate receptors and regulatory proteins, and regulate access to the underlying lipids. Hickenbottom et al., (2004) and Chong et al., (2011) show that the C-Terminal of PLIN 3 and PLIN 2 contain APOE like domains. All things considered, it seems that these APO-like proteins are a group of evolutionarily conserved proteins involved in fat storage and trafficking as well have evolved the ability to target intracellular or extracellular lipid-filled structures covered with a phospholipid monolayer.

Yang et al., (2012) studied the evolutionary relationship among 61 APO-like proteins, from seven species and grouped them based on protein similarity using Molecular Evolutionary Genetics Analysis software. The results showed genes clustered into six groups, each group including at least one human APO. Fascinatingly in the first group, four human PLIN family proteins were similar to the *Caenorhabditis elegans* APOs. In the second group, *Drosophila* Lsd2 showed similarity to 7 mammalian APOL subfamily proteins. Ten plant oleosin proteins showed similarity to mammalian APOD and APOH in group 3. Interestingly, the bacterial MLDS (RHA1 ro02104) was found to be close to mammalian APOC-I/APOC-II in group 4, indicating that APO-like proteins maybe evolutionarily older than Perilipin family of proteins. Plant fibrillarin (FIB) family proteins have been found in the plastoglobules of *A. thaliana* and have no enzymatic activity. These seven FIB proteins were clustered with four plant caleosin proteins, *Drosophila* Lsd1, and mammalian APOA-I in group 6. However, sequence alignments showed that sequence similarity among different groups is very low. Therefore, their apparent similarity may be due more to the similarity of their predicted structures than to similarity of amino acid sequences. Based upon the information provided one can logically speculate that these APO-like proteins may be evolutionarily conserved as the structural proteins of lipid droplets.

Overview of eukaryotic lipid metabolism

To better understand lipid droplets and associated proteins, one has to consider the cellular biochemical context. Therefore, I have tried to provide a brief overview of some of some of the relevant biochemical pathways involved in the synthesis and degradation of fat. One could dedicate several books to the biochemistry of lipid metabolism and that would beyond the context of this thesis I will try to focus only the most relevant parts need to show the role of LDs in regulating lipid metabolism. Tissues store triacylglycerols in lipid droplets when exogenous fatty acids are plentiful and available for import and esterification. Cellular LDs emerge from the endoplasmic reticulum (ER) and LD formation is coupled to the synthesis of neutral lipids, predominantly triacylglycerides (TAGs) and sterol esters (SE).

To make things simplified we can begin with very first biochemical building blocks produced after glycolysis acetyl-CoA. The first step of fatty acid (FA) synthesis requires the conversion of acetyl-CoA to malonyl-CoA. Acetyl-CoA is also used as the primary building block for the synthesis of cholesterol via the mevalonate pathway. Fatty acids are stored for future use as triacylglycerides

in all cells, but primarily in adipocytes. TAGs constitute molecules of glycerol to which three fatty acids have been esterified. Predominantly saturated fatty acids are found in TAGs. The major building block for the synthesis of TAGs, in cells other than adipocytes, is glycerol. Adipocytes lack glycerol kinase, therefore, dihydroxyacetone phosphate (DHAP), produced during glycolysis, is used as a precursor for TAG synthesis in adipocytes. This means that adipocytes must have glucose to oxidise for them to store fatty acids in the form of TAGs. DHAP can also be used as a backbone precursor for TAG synthesis but does so to a much lesser extent than glycerol in cells other than adipocytes. The incorporated fatty acids into TAGs require activation to become acyl-CoAs, this is dependent on the action of FA synthase. FA synthase elongates acyl-chains, stepwise, to form palmitic acid. Medium and long chain FAs are then activated to acyl-CoA by long-chain Acyl-CoA synthetase (ACSL). Several acylation steps along glycerolipid biosynthetic pathways, mediated through acyltransferases GPAT, AGPAT, and DGAT, incorporate fatty acyl-CoA to eventually form TAG. LD storage prevents FA accumulation and lipotoxicity. Both TAG and CE are stored within LDs (Wang, 2016). LD expansion also requires the phospholipid content to also increase. This is likely critical during the initial stages of the LD growth process, in which the nascent structures grow rapidly. Major phospholipids in yeast and mammals include phosphatidylcholine (50-60%) followed by phosphatidylethanolamine (20–30%) (Tauchi-Sato et al., 2002; Bartz et al., 2007). There are two major pathways that contribute to phosphatidylcholine synthesis: the Kennedy pathway for de novo synthesis of phospholipids and the Lands cycle. A short overview of the Kennedy pathway has been incorporated into the diagram below.

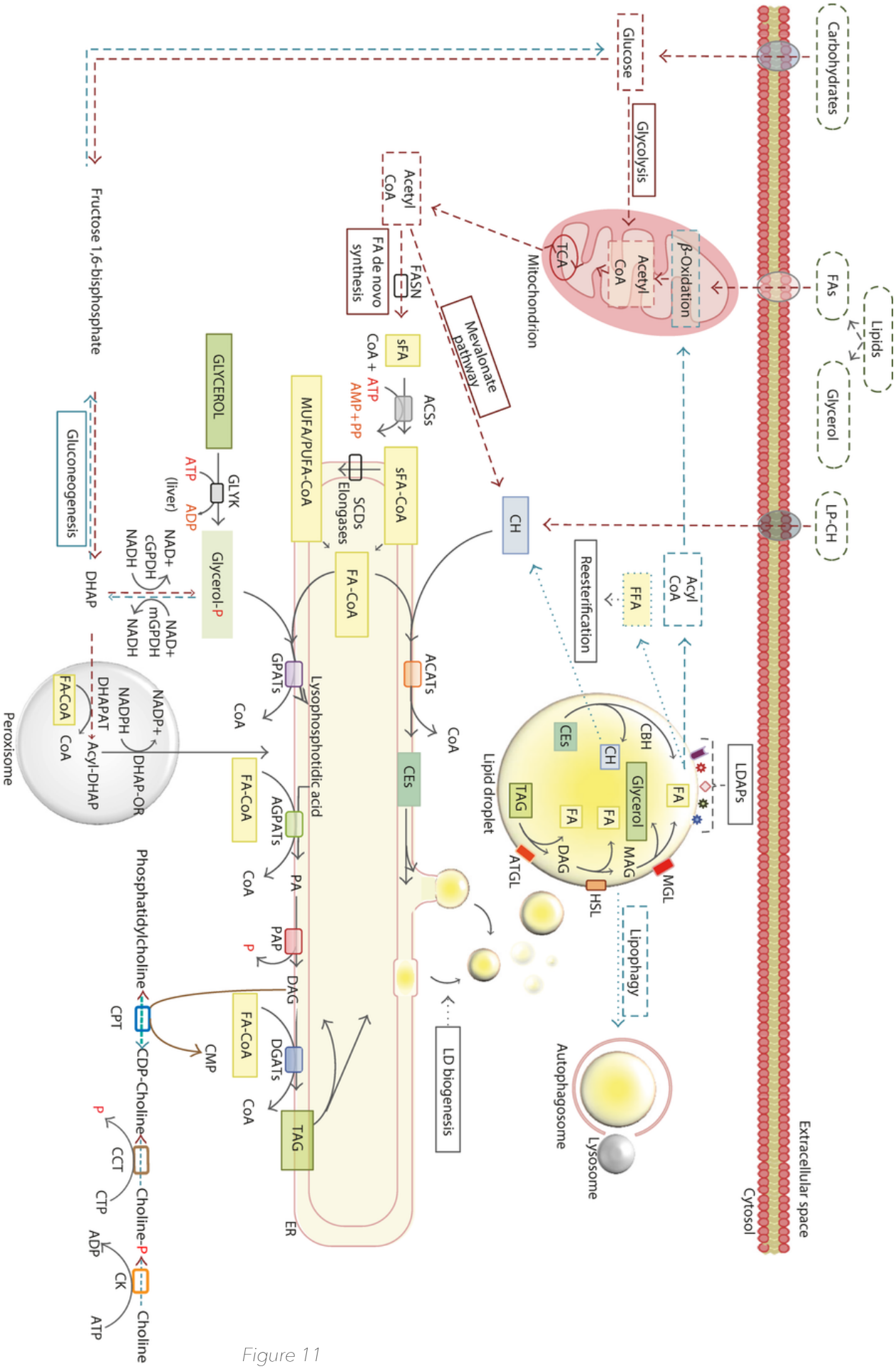

Carbohydrates
Glucose
Glycolysis
Fructose 1,6-bisphosphate
Gluconeogenesis
Lipids
FAs
Glycerol
LP-CH
Mitochondrion
TCA
Acetyl CoA
β-Oxidation
Acetyl CoA
FA de novo synthesis
FASN
Mevalonate pathway
sFA
CoA + ATP
AMP+PP
ACSs
MUFA/PUFA-CoA
SCDs Elongases
sFA-CoA
FA-CoA
CH
Reesterification
FFA
Acyl CoA
LDAPs
Extracellular space
Cytosol
GLYCEROL
GLYK
ATP
ADP
(liver)
Glycerol-P
DHAP
NAD+
cGPDH
NADH
mGPDH
NADH
NAD+
GPATs
CoA
Lysophosphotidic acid
ACATs
CoA
CES
Lipid droplet
CES
CBH
CH
Glycerol
FA
TAG
FA
FA
DAG
MAG
ATGL
MGL
HSL
Lipophagy
Autophagosome
Lysosome
LD biogenesis
ER
Peroxisome
FA-CoA
CoA
NADP+
NADPH
DHAPAT
Acyl-DHAP
DHAP-OR
FA-CoA
CoA
AGPATs
PA
PAP
P
DAG
FA-CoA
CoA
DGATs
TAG
Phosphatidylcholine
CPT
CMP
CDP-Choline
P
CCT
CTP
ADP
Choline-P
CK
ATP
Choline

Figure 11

The diagram here is a schematic overview of the biochemical pathways involved in triacylglycerol synthesis and lipolysis. Furthermore, a simplified lipid droplet with related proteins is also included. The scheme was adopted and modified with permission from Tirinato et al., (2017). The abbreviations shown in the scheme are as follows: FA-coA and MUFA/PUFA-coA are in general referred to as FA-CoA. AMP: adenosine monophosphate; ATP: adenosine triphosphate; ACATs: acyl-coA cholesterol acyltransferases; ACS: acyl-coA synthetase; AGPATs: 1-acyl-glycerol-3-phosphate acyltransferases; ATGL: adipose tissue triacylglycerol lipase; CEH: cholesteryl ester hydrolase; CEs: cholesteryl esters; CoA: coenzyme A; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; DHAP: dihydroxyacetone phosphate; DHAP-OR: dihydroxyacetone phosphate oxidoreductase; DHAPAT: dihydroxyacetone phosphate acyltransferase; ER: endoplasmic reticulum; FA: fatty acid; FA-CoA: fatty acyl-coenzyme A; FFA: free fatty acid; sFA: saturated FA; FASN: fatty acid synthase; GLYK: glycerol kinase; GPATs: glycerol-3-phosphate acyltransferases; cGPDH: cytosolic glycerol-3-phosphate dehydrogenase; mGPDH: mitochondrial glycerol-3-phosphate dehydrogenase; HSL: hormone-sensitive lipase; LDAPs: lipid droplet-associated proteins; LP-CH: lipoprotein involved in transporting cholesterol; MAG: 1-acylglycerols; MGL: monoacylglycerol lipase; MUFA: monounsaturated FA; NAD: nicotinamide adenine dinucleotide; NADH: reduced nicotinamide adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate; P: phosphate; PA-P: phosphatidic acid; PAP: phosphatidic acid phosphatase; PUFA: polyunsaturated FA; SCDs: stearoyl-CoA desaturases; TAG: triacylglycerol; TCA: tricarboxylic acid cycle.

The next important aspect to consider the lipolytic or rather catabolism of the stored fats from LDs. In this aspect, the role of the Perilipin family of proteins has been thoroughly studied. As one can already speculate the release of metabolic energy from fatty acids, is controlled by a complex series of interrelated cascades that result in the activation of triglyceride hydrolysis. My focus is not really on the generation of ATP through beta and alpha-oxidation but rather the focus is on the enzymatic cascade involved in the release of free fatty acids from LDs. In terms of the enzymatic cascade, the primary intracellular lipases are adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Other important pathways involved in LD degradation include the lipoautophagic pathway, for this reason, another important group of enzymes to consider are the lysosomal acid lipases. Although from an enzymatic point of view lipolysis is relatively simple, the regulation behind is rather complex. I will discuss more details regarding the catabolic process of lipids as perilipins play a central role in regulating enzymatic access.

Perilipin as a regulator of lipolysis

In a fed state the increased fatty acid availability allows for delivery of free fatty acids to adipocytes and insulin promotes glucose uptake to support triacylglycerol synthesis. When extracellular fatty acid supplies dwindle e.g. when energy is required for the exercise or in a fasting state, hormones initiate signalling cascades that increase kinase activity to activate lipolytic pathways in adipocytes. As discussed in previous sections, PLIN1 is one of the major targets of hormonal activation cascades. Intracellular signalling leads to the subsequent phosphorylation of perilipins, lipases, and cofactors for lipases initiates the translocation of lipases from the cytoplasm to lipid droplets and enables protein-protein interactions to assemble the lipolytic complex on the Perilipin scaffold surrounding lipid droplets. The final scaffold allows the lipases to gain access to lipid substrates

and lipolysis of stored triacylglycerols follows (Duncan et al., 2007; Lafontan and Langin, 2009). Lipolysis is catalysed by lipases that cycle between the cytoplasm or cytoplasmic surfaces of the endoplasmic reticulum and the surfaces of lipid droplets.

To better understand the formation of the lipolytic cascade we first need to know the individual proteins involved. A very important enzyme known as ATGL belongs to the family of patatin domain-containing proteins and it preferentially hydrolyses triglycerides (Granneman et al., 2007; Granneman et al., 2011). The patatin domain had originally been discovered in lipid hydrolases of certain plants and had been named after the most abundant protein of the potato tuber and because some members of the family act as phospholipases, the proteins were originally named patatin-like phospholipase domain-containing protein A1 to A9 (PNPLA1–PNPLA9) (Rydel et al., 2003; Wilson et al., 2006). The expression and enzyme activity of ATGL are both under complex regulation. The expression of ATGL is regulated by peroxisome proliferator-activated receptor (PPAR) agonists, glucocorticoids, fasting, FoxO1 activation, mTOR complex 1 (mTORC1)-dependent signalling (Zimmermann et al., 2004; Kim et al., 2006; Kershaw et al., 2007; Chakrabarti et al., 2010). Increased insulin release and food intake both result in decreased expression of ATGL (Kralisch et al., 2005). The most interesting or perhaps the most well-studied control of ATGL is known to be at the post-transcriptional level. Phosphorylation of ATGL has been reported but the physiological significance of the phosphorylation hasn't been fully established (Kim et al., 2016; Ahmadian et al., 2011). To date an important regulatory mechanism for ATGL activation involves the interaction with another LD associated co-activator protein called CGI-58 in equimolar concentration, several studies show that the patatin domain within the $\alpha\beta\alpha$ sandwich fold is responsible for enzyme activity and a protein-protein interaction with CGI-58, whereas the C-terminal part of the enzyme, has a regulatory function and mediates LD interaction of the enzyme (Lass et al., 2006; Yang et al., 2010; Schweiger et al., 2008; Gruber et al., 2010). CGI-58 co-activator gene was originally identified as comparative gene identification-58. The term comparative gene identification (CGI) was coined due to the computational methods used to identify protein sequences that were highly conserved across various species and CGI-58 was originally discovered in a comparative screen of proteomes of humans and *C. elegans*. The official nomenclature for CGI-58 is α/β hydrolase domain-containing protein-5 (ABHD5; also identified as 1-acylglycerol-3-phosphate *O*-acyltransferase), owing to the presence of an α/β hydrolase domain commonly found in esterases, thioesterases, and lipases (Granneman et al., 2007; Yamaguchi et al., 2004).

The first identified and most highly characterised lipase is hormone-sensitive lipase (HSL), an enzyme with strong diacylglycerol and cholesterol ester hydrolase activity, and weaker triacylglycerol, monoacylglycerol, and retinyl ester hydrolase activity in vitro (Lass et al., 2011). HSL is highly expressed in white and brown adipose tissue, and at lower levels in a variety of tissues. The activity of Adipose HSL is controlled by two distinct mechanisms in response to hormonal stimulation: first, the enzyme is phosphorylated by cAMP-dependent PKA leading to an increase of the intrinsic enzyme activity. HSL is known to be phosphorylated at multiple sites, some sites being

more important than others for enzyme activity (Krintel et al., 2008). Phosphorylation also promotes the translocation of HSL from the cytoplasm to the surfaces of lipid droplets to gain access to substrate lipids (Holm, 2003). Besides PKA, other kinases have also been shown to phosphorylate HSL and regulate the enzyme's activity. The list includes extracellular signal-regulated kinase, glycogen synthase kinase-4, Ca^{2+}/calmodulin-dependent kinase II, and AMP-activated kinase (Olsson et al, 1986; Garton et al., 1989). Second, phosphorylated HSL interacts with the LD protein PLIN1, which itself is a target of PKA phosphorylation.

Several studies collectively showed that PLIN1 is a strategic scaffold at the LD surface for the coordinated assembly and disassembly of multi-protein lipolytic complexes upon PLIN1 phosphorylation or dephosphorylation (Greenberg et al., 2001; Granneman et al., 2009; Miyoshi et al., 2007; Miyoshi et al., 2006). Under basal (e.g., fed or insulin-stimulated) conditions the LD-bound PLIN1 is in an unphosphorylated state, and the two major lipolytic enzymes, ATGL and HSL, are cytosolic. Furthermore, in the basal state, the C-terminus of unphosphorylated PLIN1 separately recruits the co-activator CGI-58/ABHD5 to the LD, adding another layer of protection against the lipolytic activity. Thus, under basal conditions, lipases are sequestered from LDs and lipolysis is suppressed (Brasaemle et al., 2000; Su et al., 2003; Sztalryd et al., 2003; Shen et al., 2009).

Upon hormonal stimulation, adenylyl cyclase is activated via Gαs-GTP, causing cAMP accumulation, and this PKA is activated (Viswanadha and Londos, 2008). Under these conditions, PLIN1, CGI-58, and HSL are phosphorylated by PKA, with a consequent reorganisation of the LD scaffold that is essential for the greater than 50-fold activation of cellular lipolytic rates. Furthermore, the C-terminal phosphorylation of PLIN1 disrupts interaction with CGI-58 (Taylor et al., 2012). However, CGI-58 remains bound at the LD surface and the PKA mediated phosphorylation of CGI-58 enhances its ability to recruit and activate ATGL, which catalyses the initial step of lipolysis, converting TAG to DAG and releasing FAs (Greenberg et al., 1991).

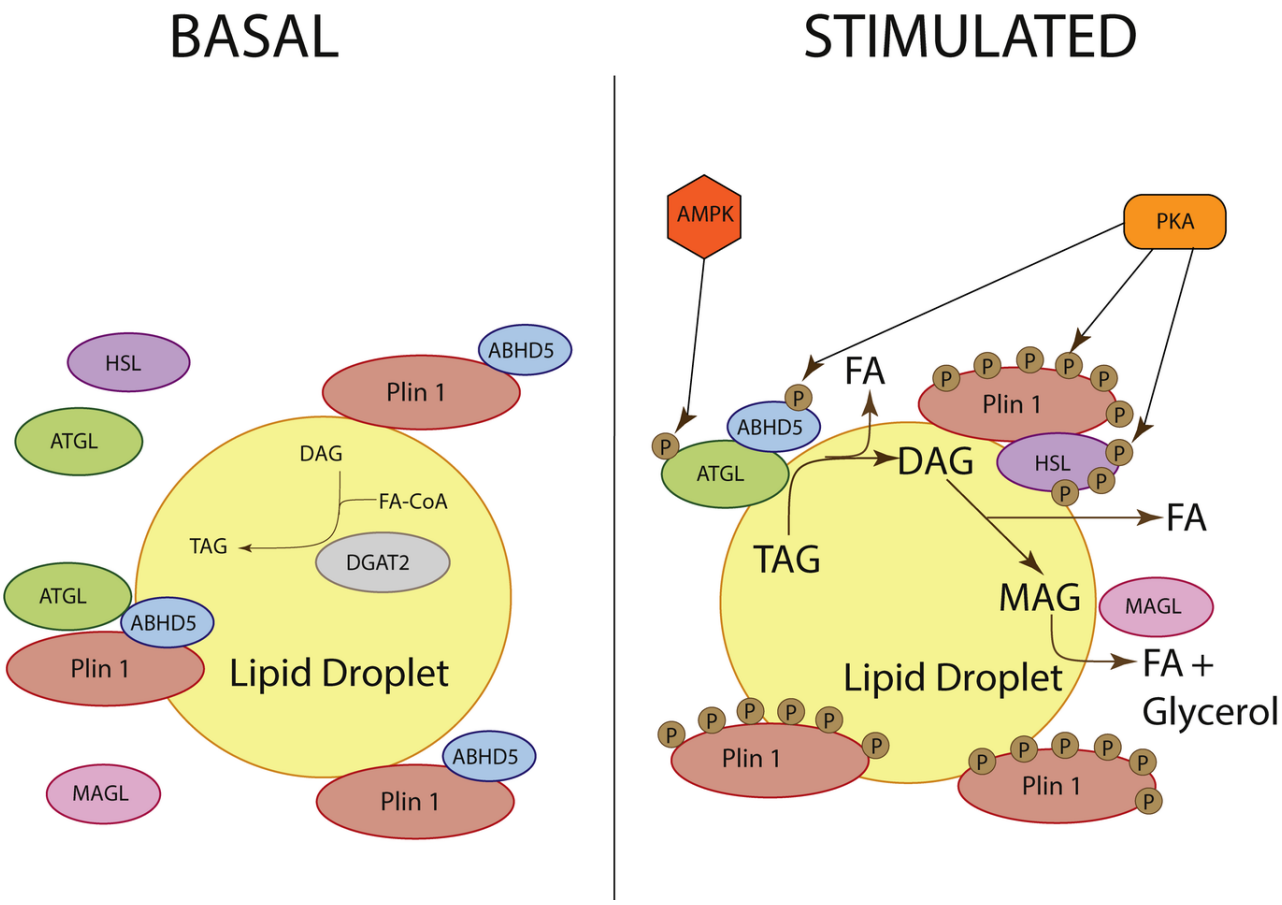


Figure 12

In the basal state, the PLIN1 is found at the surface of the lipid droplet in a complex with CGI-58/ABDH5. ATGL activity is kept quiescent through the autoinhibitory C-terminus of the lipase. Upon lipolytic stimulation through hormonal signalling, PKA, is activated and phosphorylates serine residues on PLIN1, on HSL and CGI-58/ABDH5. This results in the following; firstly CGI-58/ABDH5 dissociates from PLIN1 and recruits ATGL. Secondly, phosphorylated HSL translocates to the droplet surface and associates with PLIN1 and lipolysis commences. AMPK based phosphorylation has not been well studied but some studies do provide evidence for a role in lipid metabolism. Image taken and modified from Sztalryd and Brasaemle, (2017) with permission.

I would like to emphasise that I have only exemplified Perilipin 1 as a standard model for enzymatic lipolysis. However, this standard model is relatively simplified especially with regards to other PLINs. A great example is Perilipin 5, it binds HSL under basal conditions on LDs while restricting the lipolysis of stored triacylglycerols i.e. works as a blocker of lipolysis in a basal state (Wang et al., 2009). The C-terminal of Perilipin 5 contains overlapping binding sequences for ATGL and ABHD5. In contrast, the C-terminus of Perilipin 1 is only able to bind ABHD5 and not ATGL; moreover, the sequence of the ABHD5 binding site is not conserved among Perilipin 1 and Perilipin 5 (Wang et al., 2011; Granneman et al., 2009a; Granneman et al., 2011). Perilipin 5 is an effective protector of stored triacylglycerols from lipolysis under basal conditions while recruiting lipolytic proteins/enzymes to LDs; however, upon hormonal signalling activate PKA phosphorylates Perilipin 5 and lipolysis increases (Wang et al., 2011; Pollak et al., 2015).

Perilipin 2, as previously stated, is a major LD associated Perilipin in cells not expressing Perilipin 1 or Perilipin 5. Perilipins 1 and 5 exert specific and distinct control over lipolysis, as controlled by PKA-mediated phosphorylation of themselves, lipases, and co-factors, Perilipin 2 only plays a modest role in attenuating lipolysis and is not known to be phosphorylated by PKA. It is substantially more permissive to lipolysis than either Perilipin 1 or Perilipin 5 and does not effectively recruit lipases to lipid droplets through protein binding interactions under either basal or hormonally stimulated conditions that activate PKA. Instead, the overexpression of Perilipin 2 in cells reduces the access of ATGL to lipid droplets, thus attenuating lipolysis (Wang et al., 2011; Wang et al., 2009; Bell et al., 2008; Listenberger et al., 2007).

Accessory pathways of lipid droplet catabolism

The consumption of fats from LDs is a highly regulated process. However, with recent research, it has become evident that LDs are taken up by autophagy as an alternative route for lipid mobilisation and LD destruction (Singh et al., 2009). Autophagy, in general, is considered to be a conserved cellular pathway that delivers cytoplasmic contents to lytic compartments for breakdown and recycling in eukaryotes, full discussion into details of the mechanism behind autophagy, in general, is beyond the scope of this thesis. However, I try to provide an overview of autophagy in general, to provide an understanding of lipophagy. LD autophagy, termed lipophagy or lipoautophagy, which delivers LDs to lytic compartments for degradation, is mediated through actions of autophagic (Atg) proteins and might represent a selective type of autophagy, targeting LDs specifically for destruction. Lipophagy, together with lipolysis, plays a critical role in energy metabolism during fasting. Though these two types of pathways for fat mobilisation appear to be distinct in their molecular mechanisms, recent evidence has revealed crosstalk between them (Singh and Cuervo, 2012).

Cells defective in autophagy are unable to consume dysfunctional proteins and aged organelles during nutrient deprivation, resulting in their death. At the organismal level, autophagy is critical for physiology and development and autophagy defects have been linked to numerous human diseases. The molecular mechanisms of autophagy were revealed by genetic and biological studies in yeast and in higher eukaryotes. To date, three major types of autophagic mechanisms known macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), differing in cargo delivery, have been characterised (Yang and Klionsky, 2010; Sahu et al., 2011; Cuervo and Wong, 2014). Unlike macroautophagy that involves autophagosome formation prior to fusion with the lysosomes, microautophagy involves a direct engulfment of cytoplasmic contents by modifying membranes of lytic compartments, such as lysosomes or late endosomes (Santambrogio and Cuervo, 2011). In both cases, the resulting internal vesicles in the lumen are subsequently degraded by hydrolases within lytic compartments. By contrast, CMA involves a direct translocation and degradation of cytosolic proteins in lysosomes (Cuervo and Wong, 2014). However, only macroautophagy and chaperone-mediated autophagy (CMA) have been shown to degrade LDs and associated proteins.

Kaushik and Cuervo, (2015) recently demonstrated that CMA can also modulate intracellular lipolysis by selectively removing PLINs from discrete regions from the surface of LDs. The follow up by Kaushik and Cuervo, (2016) revealed the requirement of an AMPK-mediated phosphorylation event in the selective degradation of PLINs and subsequent triggering of lipolysis. The group further showed that the dependence on AMPK activity for the degradation of PLIN2 by CMA and found that degradation of PLIN2 is required prior to lipolysis. Activation of AMPK facilitates both the priming of a subset of PLIN2 molecules for their removal by CMA as well as activation of downstream lipolytic mechanisms, such as the described AMPK-dependent stimulation of macroautophagy and fatty acid oxidation (Long and Zierath, 2006).

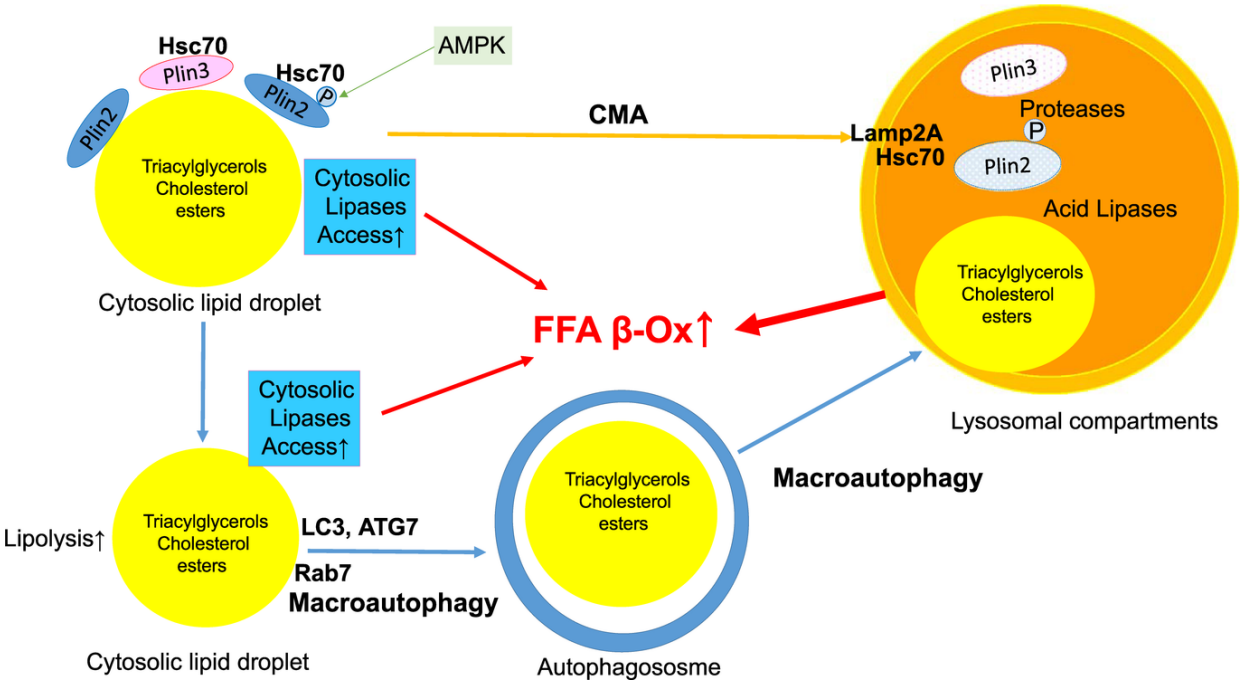


Figure 13

Schematic representation of autophagy leading to lipid droplet degradation used with permission from Sztalryd and Brasaemle, (2017). PLINs provide a so called autophagic barrier and removal of PLINs from the LD surface is known to be mediated by CMA. AMPK may play a role in priming the protein for CMA. After PLINs are tagged with Hsc70 the process of Lamp-2A based recognition and eventual lysosomal degradation. The removal of PLINs leaves the LD helpless against not only classical lipases but also allows the increased binding of autophagy effector proteins to the LD surface. This leads the LD to be sequestered by autophagosomes. Autophagosomes eventually fuse with lysosomes to form autolysosomes where lysosomal lipases degrade triacylglycerols into free FAs.

Trichoplax adhaerens as a model organism

In the Phylum Placozoa, *Trichoplax adhaerens* is the only named species (with 19 reported lineages (Eitel et al., 2013)) and so far, it is considered to be one of the simplest metazoan organisms containing only six types of cells without any kind of mesenchymal tissue (Ringrose et al., 2013; Smith et al., 2014). The species are found inhabiting pelagic marine environments around

the world within a temperature range between 10°C - 32°C and show seasonal variations such as higher abundance in summer. Analysis of all available data identified Placozoa as the most basal diploblast group (Schierwater et al., 2009) and placozoan morphology (disk-shaped and lacking symmetry) fits the placula hypothesis of metazoan evolution proposed by Otto Bütschli in 1884, requiring only minor differentiation processes (Schierwater et al., 2009a).

In terms of Placozoan morphology, it is a disk-shaped animal built by six different cell types and locomotes by ciliary gliding. The body can be divided into a ventral plate made up of ventral ciliated epithelial cells with microvilli, cells containing large lipophilic inclusions (lipophil cells) and gland cells (Ruthmann et al., 1986). The dorsal surface of *Trichoplax* is bounded by a thin layer of dorsal epithelium composed of ciliated epithelial cells, that together with the ventral plate, surround crystal cells containing birefringent crystals. Fiber cells contact other cell types through their branching processes (Smith, 2014). Stem cells are thought to be present around the margins of the whole animal, however, they haven't been reported morphologically (Martinelli and Spring, 2004; Jakob et al., 2004). One can also observe shiny spherical dark granules, which are probably found on the dorsal side and may represent lipid inclusions but may also be involved in defence mechanism against predators (Jackson et al., 2009). Although little is known about the functions of the 6 described cell types it is known that the animal feeds by external digestion underneath its ventral plate, ventral epithelial cells are thought to be engaged in nutrient uptake are thought to resemble cells of the digestive tract because of the presence of microvilli (Halanych, 2004). The gland cells are now proposed to be neurosecretory, rather than digestive enzymes secretors (Halanych, 2004; Smith et al., 2014).

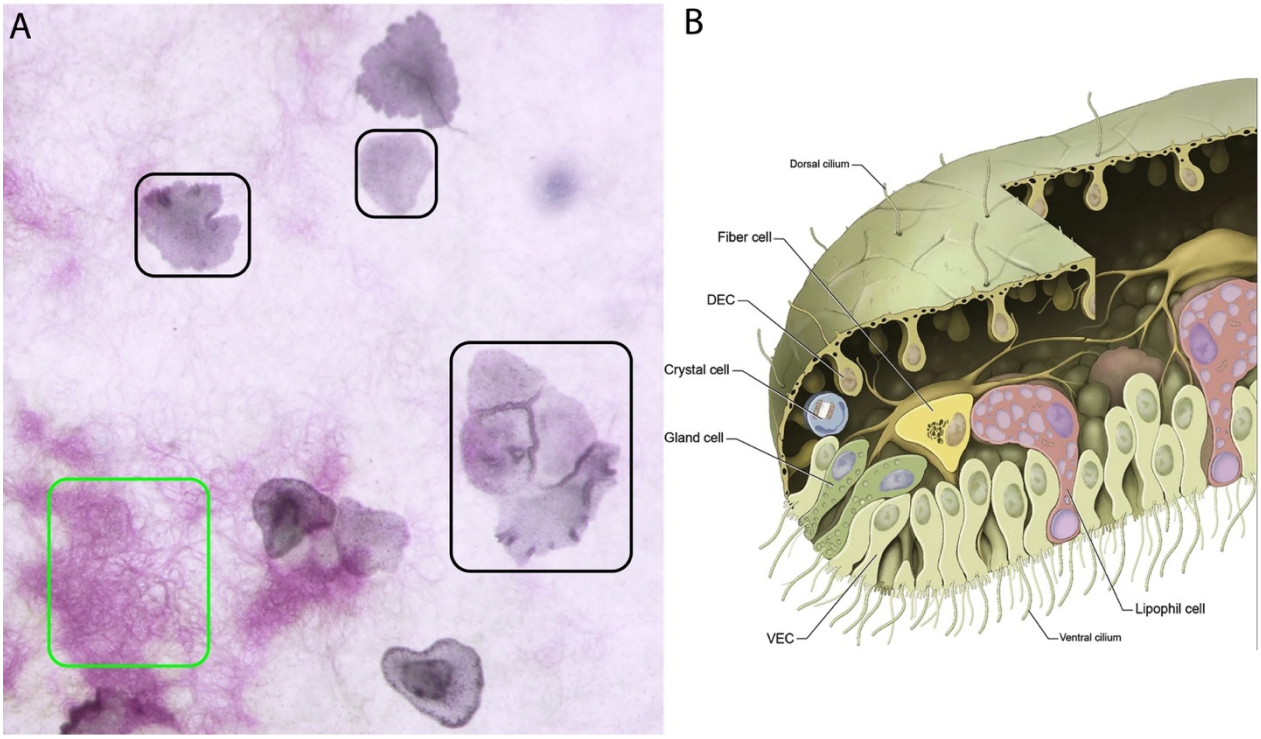


Figure 14

Laboratory culture and Illustration of *Trichoplax adhaerens*. A) Shows the multiple individuals of *Trichoplax adhaerens* feeding on pink coloured mix culture of algae and *cyanobacteria* labelled by the green box. Individual animals have been marked with black boxes. B) Drawing of *Trichoplax adhaerens* body plan. Shiny spheres are not depicted and are probably located in dorsal epithelial cells (DEC). Ventral epithelial cells (VEC) are ciliated and possess numerous microvilli. Reprinted from Smith et al., (2014), with permission.

In a laboratory environment, *T. adhaerens* usually reproduces by binary fission, however, development of embryos until a certain stage has been observed (Eitel et al., 2011). Despite the presence of embryos sexual reproduction has never been observed, molecular analysis revealed allele shuffling, indicating sexual reproduction in this species (Signorovitch et al., 2005). The genome of *Trichoplax* was sequenced and published back in 2008 and contains approximately 98 million base pairs with over 11,500 protein-coding genes (Srivastava et al., 2008). Genomic analysis also reveals large blocks of conserved synteny relative to vertebrate genomes. Furthermore, an extensive set of transcription factors associated with metazoan development (e.g. patterning), as well as differentiation and cell type specification, such as essential components of TGF-ß signalling, GATA family zinc-finger transcription factors that are involved in cardiac and blood cell fates in vertebrates, can be found. Moreover, the genome contains several genes coding for putative ECM proteins, despite the fact that no extracellular matrix (ECM) was identified to date.

Taken together, it is extremely fascinating, that the placozoan genome codes for and transcribes several genes associated with 'higher' animals without apparent correlate present in the animals' morphology (Schierwater et al., 2009).

With regards to NRs, the receptors and their network have mainly been studied in more complex organisms already in possession of an extensive endocrine network. Genomic analysis of *Trichoplax adhaerens* has already revealed a possibility of four highly conserved nuclear receptors, namely orthologues of HNF4 (NR2A), RXR (NR2B), ERR (NR3B) and COUP-TF (NR2F). Despite the fact that this basal metazoan only contains four NRs, the study of NRs in *Trichoplax* presents an interesting challenge due to lack of versatile methods and analytical techniques proven to work in a controlled laboratory setting such as effective RNAi, GFP-labelling and CRISPR/Cas9 based gene modification.

Caenorhabditis elegans as a model organism

Caenorhabditis elegans is rather a small (about 1mm in length) well differentiated free-living soil organism. It belongs to the phylum *Nematoda* it can feed on bacteria as a source of nutrition, it can be easily cultivated in large numbers in a laboratory environment and finally having a transparent body type makes it even more of an attractive model system for microscopy. Using *C. elegans* as a model organism for studying molecular and developmental biology research started in the early 60s. Sydney Brenner was the first scientist to propose using *C. elegans* as a model organism for studying biology (Brenner, 1974). By providing a robust yet easy system to investigate the very fundamental processes of physiology and anatomical development several Nobel prizes have been given to scientist studying biological events in *C. elegans*. The first was awarded to

Sydney Brenner, John Sulston and Robert Horvitz in medicine or physiology for genetics and organ development and programmed cell death. The second went to Craig Mello and Andrew Fire for discovering the mechanism of RNA interference. interference. A Nobel prize in chemistry for the discovery and development of green fluorescent protein is associated with *C. elegans*.

In 1998, the whole genome sequence of *C. elegans* was completed (*C. elegans* Sequencing Consortium, 1998), the size of the genome is relatively small compared to that of human, it is about 100 million base pairs long. The complete sequence meant quick and easy access to genetic information. Even though the absolute size of the human genome is much larger than the worm's genome, it still codes for about 20,000 genes, the 100 Mb worm genome is 30 times smaller (International Human Genome Sequencing Consortium, 2004). Consequently, about 26% of the worm genome is exonic, compared to 1-2% of the human genome (Dupuy, 2004). In addition, the majority of intergenic regions are less than 2kb, and introns with a 65 bp median length are much shorter, compared to humans where the median length of introns is about 3 kb (Spieth, 2006).

One of the very interesting features of *C. elegans*, which is in a way advantageous over similar animal systems like *Drosophila*, is its deterministic cell development. The fate of each cell is more or less genetically predefined, each larval stage, as well as the adult worm, has the same number of somatic cells. As far as the sex of the organism is concerned, it can be either found as a hermaphrodite or as a male. *C. elegans* has five pairs of autosomes and a single pair of sex chromosomes (hermaphrodites) (Costa et al., 1988). The sexual determination in *C. elegans* is similar to that of *Drosophila*, so that the ratio of sex chromosomes to autosomes determines the sex of the animal. A XX combination of the 6th chromosome pair will produce a hermaphrodite while a XO combination will produce a male. The most common sex in nature is the hermaphrodite. When males mate with hermaphrodites they tend to produce progeny that is 50% male, this is actually a useful and practical phenotype as it makes checking for positive crosses easier. A hermaphrodite contains 959 somatic cells and the male sex contains 1031 somatic cells (with a few exceptions) (Wood, 1988). The worm has a relatively high fecundity rate producing approximately 300 to 350 offspring per hermaphrodite. The figure below represents the typical anatomy of a hermaphrodite *Caenorhabditis elegans*.

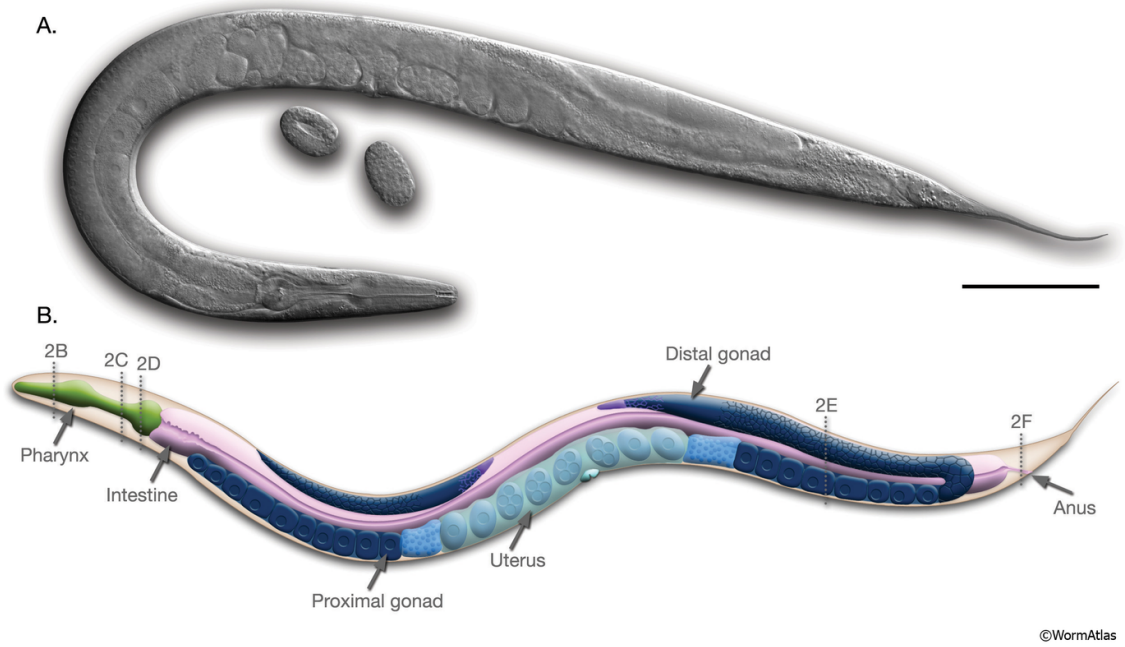


Figure 15

Anatomy of an adult hermaphrodite *Caenorhabditis elegans* used under Creative Common Licence from **www.wormatlas.org**. A) Differential interference contrast image of an adult hermaphrodite, left lateral side. Scale bar 0.1 mm. B) Schematic drawing of anatomical structures as seen from left lateral side.

With regards to metabolic homeostasis, *C. elegans* maintains its energy balance using many physiological processes that include neuroendocrine signalling, storing, mobilising and utilising energy stores (Ashrafi, 2007). The worm is not known to have specialised adipocytes, however, it is capable of storing its body fat mainly in the form of triglycerides, which is similar to the mammalian body fat (Mullaney and Ashrafi, 2009). Several authors have revealed using whole animal staining assays with lipophilic dyes that *C. elegans* body fat is mostly stored in the intestinal and skin-like hypodermal cells (Mullaney and Ashrafi, 2009; Mak, 2012). Despite the lack of tissue specialisation and compartmentalisation that exist in mammalians, many essential pathways required in mammals can also be found phylogenetically preserved in *C. elegans* to some degree; examples include, insulin-like signalling, serotonin signalling, TOR signalling and core metabolic pathways including mitochondrial β-oxidation and fatty acid synthesis, elongation and desaturation (Apfeld and Kenyon, 1999; Srinivasan et al., 2008; Soukas et al., 2009; Van et al., 2005; Watts and Browse, 2002).

The life cycle of *C. elegans* is short and temperature dependent. After embryogenesis, which partially takes place in the hermaphrodite body and partially outside, the L1 larva hatches. In the presence of food, the worm should go through 4 larval stages (L1–L4) and finally develops into an adult worm. After which the cycle repeats.

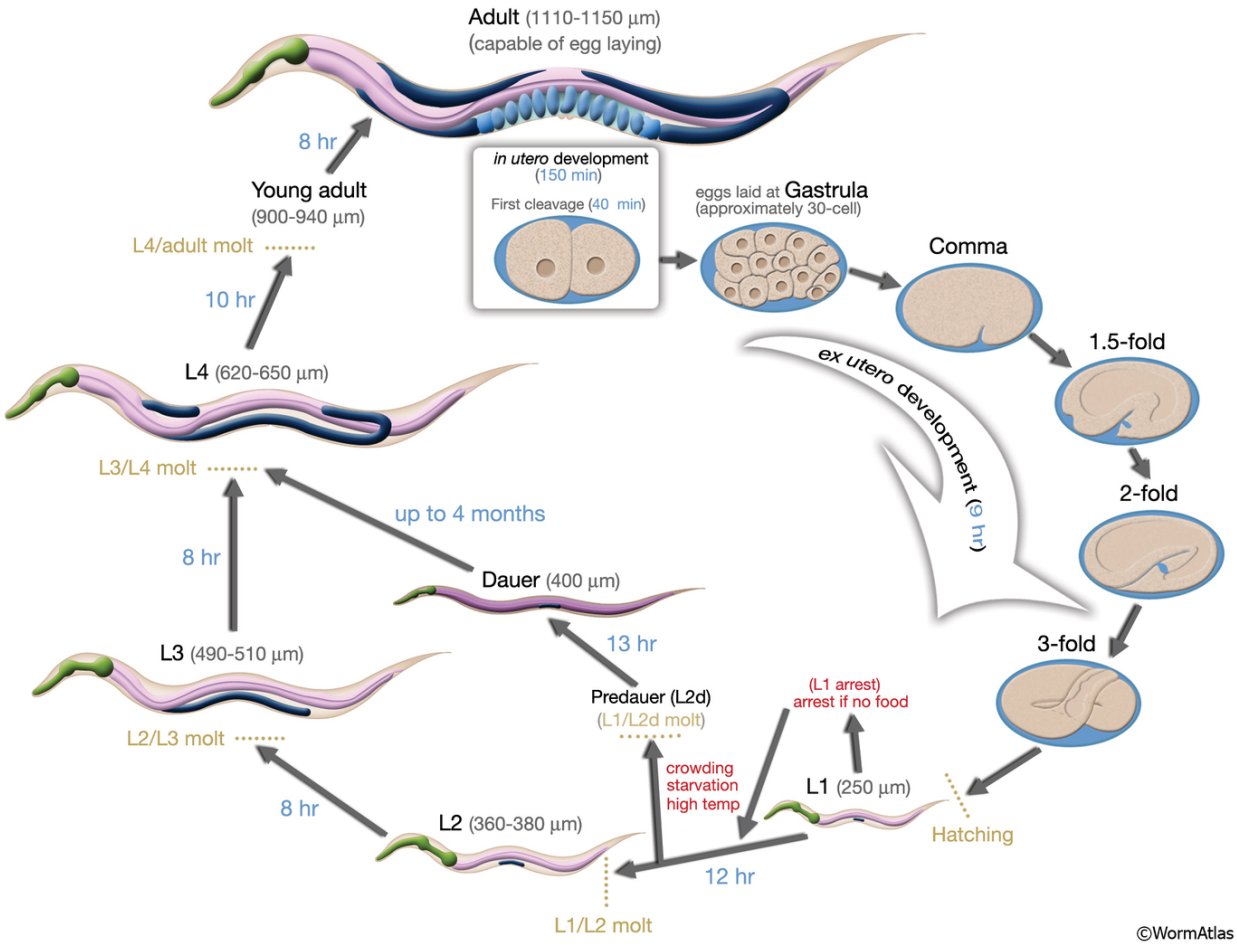


Figure 16

Life cycle of *Caenorhabditis elegans* at 22°C. 0 min is fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. First cleavage occurs at about 40 min. post-fertilization. Eggs are laid outside at about 150 min. post-fertilization and during the gastrula stage. The length of the animal at each stage is marked next to the stage name in micrometers (µm). Figure taken from **www.wormatlas.org** and used under Creative Common Licence. (Altun and Hall, 2009.).

As demonstrated in the figure above, if the conditions are unfavourable, the larva might enter the dauer stage. Animals in the dauer stage are very resistant to miscellaneous types of stresses. Genes that regulate entry into the dauer phase are strongly linked to ageing and longevity (Inoue et al., 2007; Hu et al., 2006). Larval development can even be arrested at the L1 stage (Baugh, 2013). The normal life span is around 2–3 weeks but under laboratory conditions, the lifespan may be longer and it depends on conditions (temperature, food availability, etc.).

Study reasoning and strategy

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. 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 (Vohanka et al., 2010; 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 (Sato et al., 2004; Beyer et al., 2007; Wiederhold et al., 2004; Lu et al., 2006; Huang et al., 2012a). 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

This section of the dissertation is compiled using three different original research publications namely Novotný et al. (2017); Chughtai et al. (2015); Kostrouchová et al. (2017). In order to avoid unnecessary repetition, I have summarised the underlying principles of the techniques and methods employed in all of the above-mentioned publications. For specific details on the procedures, I refer to the original articles themselves.

Animal culture, strains, transgenic lines and genome editing

In three publications, various strains of the nematode species *C. elegans* were used. 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 used in the conducted experiments were requested from other research groups and a number of them were created through genetic engineering techniques namely CRISPR/Cas9 technology.

Transgenic lines were prepared using microinjections into gonads of young adult N2 hermaphrodites as described by Tabara et al., (1999); Timmons et al., (2001); Vohanka et al. (2010). Most injections also included mCherry co-injection markers: pCFJ90, pCJ104 and pGH8 Dickinson et al., (2013).

Regarding *Trichoplax* culture, the animals were cultured in Petri dishes containing filtered artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) with a salinity of approx. 38–40 ppt. *Rhodomonas salina* (strain CCAP 978/27), *Chlorella sp.*, *Porphyridium cruentum* (UTEX B637) 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 the stock. The cultures were kept at approx. 23°C and an automated illumination for 12h/day was used with a conventional light bulb on a daylight background from late spring to mid-summer in the laboratory located at 50.07031N, 14.42934E with laboratory windows oriented eastward. The natural illumination included almost direct morning light from 8 AM to 10:30 AM, indirect sunlight for most of the daytime and sunlight reflected from a building across the street from 1 PM to 6 PM. Algae were maintained as described (Kana et al., 2012; Kana et al., 2014). The experiments were performed predominantly during sunny weather.

Principle and implementation of CRISPR/Cas9 technology in *C. elegans*

The discovery and implementation of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system for genetic engineering has completely revolutionised the study of gene function not only in *Caenorhabditis elegans* but also in other organisms (Wang et al., 2016). The system allows for the creation of precisely targeted mutations in endogenous genes, this allows investigators to examine the relationship between gene function and phenotype. By inserting desired coding sequences for a marker or fluorescent protein, the expression and localisation of

endogenous proteins can be monitored. The biggest caveats of classical methods, such as extra-chromosomal arrays have been unintended over-expression and silencing. Reporter gene fusions' versatility has dramatically been enhanced. By inserting a fluorescent protein (FP) into the endogenous locus one can use phenotypic assays to access the viability and functionality of the resulting fusion protein. Taken together, these advantages allow for more control over experiments and consequently greater confidence in the results obtained. An added benefit, apart from having transgenes or intended modifications being integrated into the genome, is the fact that the CRISPR/Cas9 system is technically easier to implement i.e. no need for microparticle bombardment (Praitis et al., 2001) and is universal i.e. no need to special background stains as need by the Mos1 transposon system (Robert and Bessereau, 2007; Frøkjær-Jensen et al., 2008; Frøkjær-Jensen et al., 2010; Frøkjær-Jensen et al., 2012).

There are some recent landmark publications highlighting the ease, versatility and time efficiency of the CRISPR/Cas9 system in *C. elegans*. A variety of different approaches have been developed for *C. elegans* and in general, all of these are effective to various degrees, with different strategies being best suited to depending on the experimental goal. In principle, one can now make any desired change to the *C. elegans* genome in a matter of weeks. Here I provide a summary of the principle of the technique based on the following publications Dickinson et al. (2013); Dickinson et al., (2015); Arribere et al., (2014); Zhao et al., (2014); Paix et al., (2015); Dickinson and Goldstein, (2016); Ward, (2015).

The Cas9 is an endonuclease that can be found in Archaea and bacteria where it has evolved as an immune defence against phages and plasmids (Hsu et al., 2014). Restriction endonucleases' protein structures recognise particular DNA sequences e.g. *Bam*H I binds DNA at 5'-GGATCC-3', however, the specificity of Cas9 is determined by the sequence of an associated small RNA molecule. In bacteria, Cas9 binds two small RNAs: a CRISPR RNA (crRNA), which determines the target specificity towards DNA and a *trans*-activating CRISPR RNA (tracrRNA) that base pairs with the crRNA and activates the Cas9 enzyme. For practical reasons one fuse the two to generate a chimeric *single guide RNA* (sgRNA) that allows Cas9 cleavage of DNA substrates (Jinek et al., 2012). The 20 base pair *guide sequence* at the 5′ end of the sgRNA directly responsible for the sequence cleaved by the endonuclease. The binding is based on the Watson–Crick base pair principle. Apart from having the bases paired with the target DNA, the enzyme has to interact with a *protospacer-adjacent motif* (PAM) on the target DNA strand. The Cas9 endonuclease found in *Streptococcus pyogenes* is able to recognise the PAM sequence NGG (where N can be any base). To-date *Streptococcus pyogenes* Cas9 is the most frequently used for experimental research. The Cas9 endonuclease from *Streptococcus pyogenes* can be essentially programmed to cleave any desired nucleotide sequence that contains a GG dinucleotide, by simply modifying the sequence at the 5′ end of the sgRNA. The ease of programming is what makes Cas9 such a powerful and versatile tool for genome engineering.

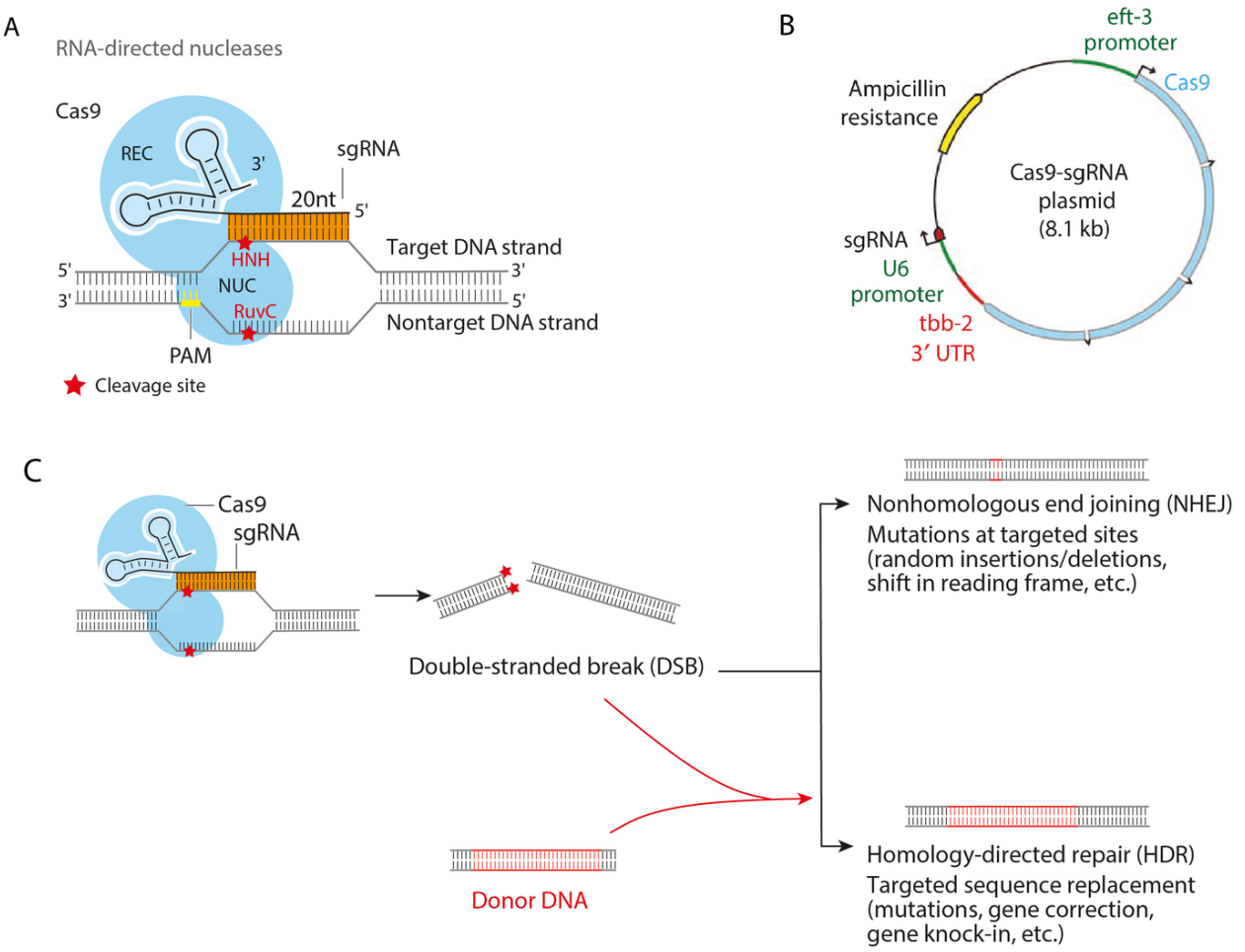


Figure 17

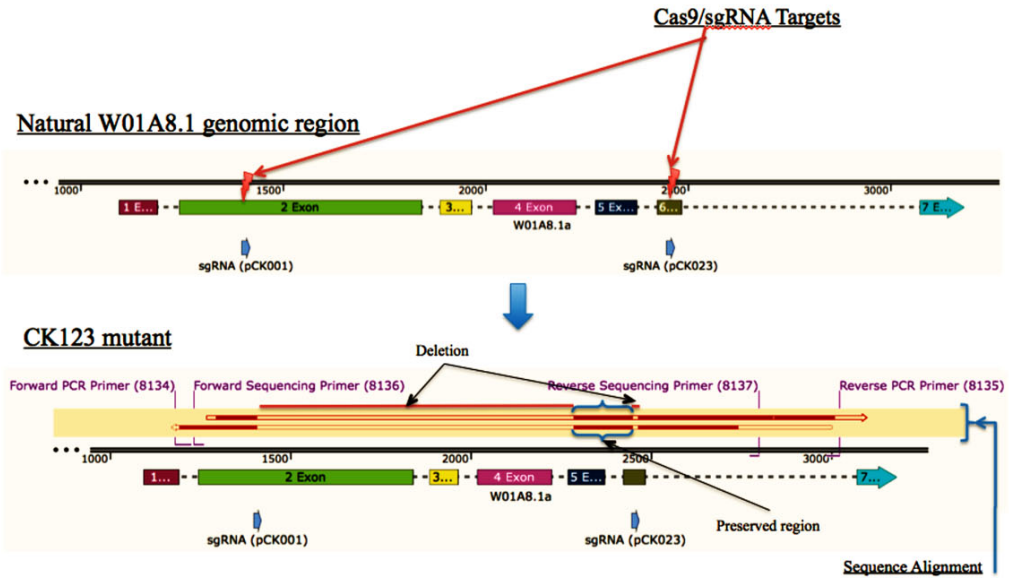
Outline of the basic working principle of the CRISPR-Cas9 system. A shows the Cas9 endonuclease and its relationship between sgRNA and target DNA. It recognizes its target DNA through approximately 20 nucleotide base-pairing interaction between a single guide RNA (sgRNA) and the target DNA strand. Cas9 also interacts with the protospacer-adjacent motif (PAM) of its DNA target through its PAM-interacting domain at its C-terminus. Cas9 uses its two nuclease domains (HNH and RuvC) to cleave the double-stranded DNA, creating a DSB. The HNH, RuvC, and PAM-interacting domains, as well as an evolutionarily divergent wedge domain (WED), all reside in the Cas9 nuclease (NUC) lobe. B represents the plasmid used throughout our experiments and was requested developed by Dickinson et al., (2013). The plasmid contains a full Cas9 endonuclease gene with C. elegans specific promoter and UTR regions from efficient expression. The plasmid also contains a region for expressing sgRNA driven by a C. elegans RNA polymerase III promoter. C demonstrates the possible outcomes of Cas9 induced double-stranded breaks (DSBs). DSB can be repaired in two possible ways, one is known as non-homologous end joining, leading to random insertions/deletions/ORF-shifts, and the other homology-directed repair, ideally ending up with specific repair based on the donor DNA. The figures were modified with permission from Wang et al., (2016); Dickinson et al., (2013).

Cas9 can generate a DNA double-strand break at a sequence-specific location within the genome. These double-strand breaks are useful because they let the researcher to make use of endogenous DNA repair machinery in the cell to generate custom modifications to the genome. Essentially two different types of DNA repair strategies have been employed to produce custom modifications in *C.*

elegans. As highlighted in the figure above, once a double-stranded DNA break is created there are two naturally occurring mechanisms involved in the repair of the break. One possibility is an error-prone repair which known as non-homologous end joining (NHEJ) and the other option is template-based recovery known as homology-directed repair (HDR). When DNA breaks occur for other natural reasons they are repaired using either of these mechanisms. By exploiting this pre-existing DNA repair system with the power of CRISPR/Cas9 directed breaks one can essentially achieve any desired genomic modification. As shown above, the NHEJ is a relatively simple way of repairing DNA after a break. Using this approach, the investigator is able to create random deletions or insertions or frame shifts, in other words, these changes lead to a gene sequence disruption when the Cas9 is directed to a specific gene sequence.

We applied the NHEJ method to create a gene disruption in the *W01A8.1* gene locus. We did this by constructing two plasmids carrying CRISPR/Cas9 system, based on Dickinson et al. (2013), each coding a sgRNA targeting the second and the sixth exon of the *W01A8.1* gene. By doing so we were able, through the process of NHEJ repair after the Cas9 induced double-stranded break, to create a large deletion. The genomic map, CRISPR/Cas9 targeted sites as well the resulting PCR amplification of the deleted segment has been shown below (Figure 18).

Panel A - Scheme of large deletion in *W01A8.1* gene



Panel B - Scheme of synthetic W01A8.1(a)synth::gfp plasmid

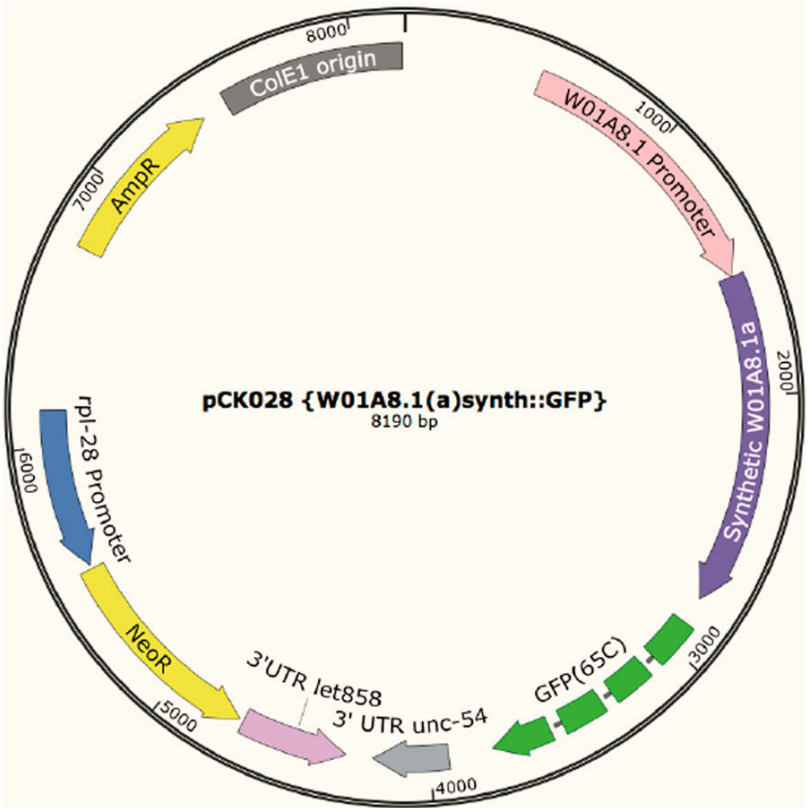


Figure 18

Generation of targeted deletions in W01A8.1 gene and selection with extra-chromosomal array. (A) shows the genomic region of W01A8.1 gene being targeted by CRISPR/Cas9 sgRNAs at two marked positions +. After selection, the mutant CK123 (bottom) was generated and the genomic segment between primers #8134 and #8135 was PCR amplified and the resulting amplicon was sequenced from both directions using

nested sequencing primers #8136 and #8137. It is clear from the sequencing alignment that a significant portion of the gene was disrupted while a small sequence, between the two sgRNA sites, was preserved. (B) represents the extra-chromosomal array used for selection with the CRISPR/Cas9 based deletion shown in (A). The plasmid is carrying a synthetic version of W01A8.1a fused to gfp and was used as a potential balancer if loss of W01A8.1 would be lethal. The plasmid was injected together with mCherry selection markers and CRISPR/Cas9 sgRNA plasmids. The sequence alignment and graphics were designed using SnapGene software.

In our experiments, we also employed the HDR pathway with the CRISPR/Cas9 System. As illustrated in the diagram above there are multiple ways that the template/donor DNA can be used in conjunction with the targeted breaks i.e. for creating specific mutations, gene corrections/modification, knock-ins, etc. Under physiological conditions, cells tend to repair a DNA break of one chromosome using the homology of the second pair. Thus, when a specific modification is needed then one can custom design a sequence of interest, that needs to be used as repair template/donor DNA, to be introduced along with the Cas9/sgRNA. Modifications present in the repair template are then copied into the genome in an error-free manner. For the HDR to work one also has to introduce in the donor DNA with a flanking homologous sequence to the region of interest. For example, when a fluorescent protein (FP) tag at the C-terminus is required then the ideal location of the beak would be near the end of the gene. The donor DNA would contain the FP sequence flanked by about 500-600bp of the gene DNA sequence at the 5' side and 500-600bp URT of the gene as the flanking sequence on the3' side. The image below illustrates the concept of flanking homology when introducing a knock-in in this FP being the desired insertion. As a note of caution the original DNA sequence to which the sgRNA was being targeted at must be altered in the donor DNA if this doesn't happen by itself.

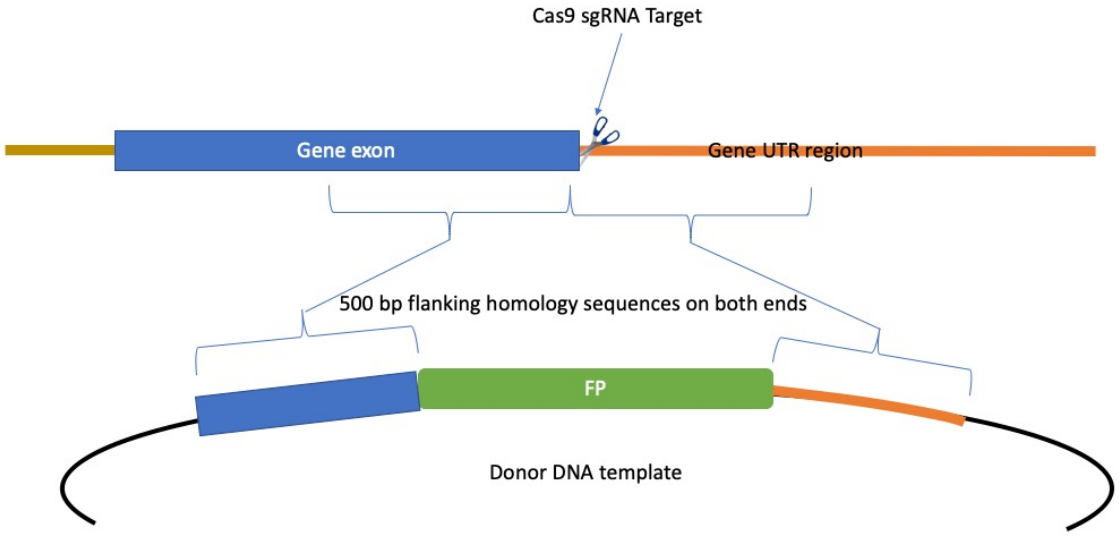


Figure 19

Illustrating the basic concept of HDR. The donor DNA contains homology arms on either side of the desired insert sequence (FP). The length of the homology arm can vary. Various publications have used different length depending on the size of the insertion sequence. Sometimes the sgRNA targeting sequence isn't at the perfect centre of the flanking region and is thus still present in either one of the homology arms. This needs to be mutagenised or modified in order to avoid Cas9 being targeted to the donor DNA.

We also implemented the HDR technique in several of our experiments. In our experimentation, we modified the *F28F8.5* gene locus based on the approach from Dickinson et al., (2015). The approach uses very large repair templates which also contain a rather large built-in positive selection system. Dickinson et al., (2015) refer to this as a Self-Excising Cassette (SEC). In the figure below the design of the SEC in relation to FP and a possible modification of the *his-72* locus has been shown as demonstrated by Dickinson and Goldstein, (2016). In the shown example when the FP-SEC-3xFlag is inserted at the N-terminus of the *his-72* gene it results in its disruption and no transcription of the gene should be possible from this locus as the promoter sequence is severed and a large foreign sequence has been inserted. The SEC contains genes and sequences that allow for positive selection (roll phenotype and hygromycin resistance) as well as heat shock inducible Cre/LoxP system. When the FP-SEC-3xFlag sequence has been inserted it represents an intermediate state where the expression of the native gene is blocked, the insert functions as a transcriptional reporter. Once the animals are heat shocked then the Cre/LoxP system is activated resulting in the removal of the SEC and the result is a fused FP with the protein of interest thus resulting in a translational fusion with a functional protein (if the FP doesn't interfere with the natural function of the protein). The technique was used to create *F28F8.5* heterozygote mutants as well as an N-Terminus fluorescent protein-tagged F28F8.5 line. The map of the genomic modification of F28F8.5 has been presented below (Figure 38). In further experimentation, we used the SEC method to also genetically modify the *W01A8.1* gene to be tagged with a C-Terminus FP.

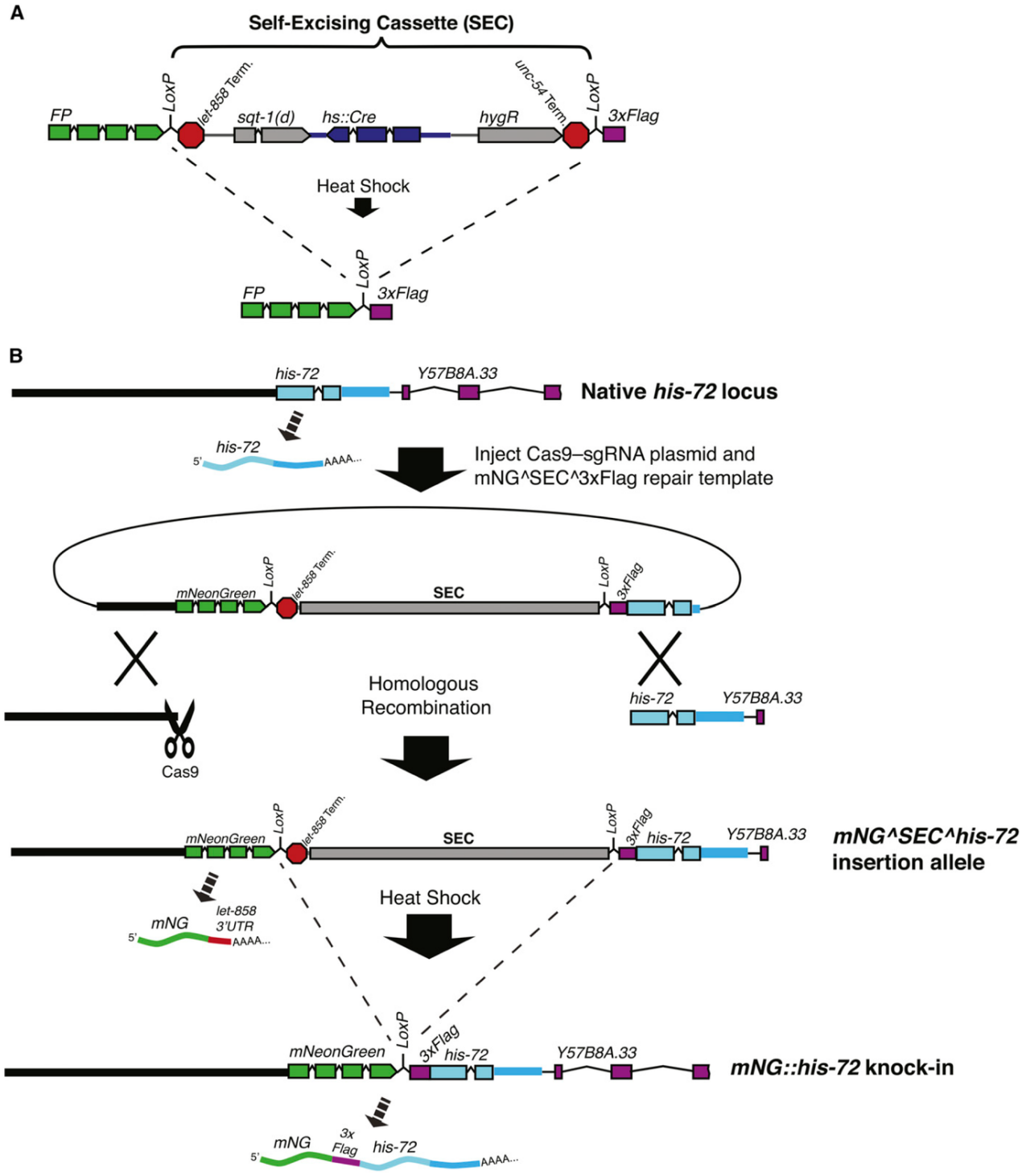


Figure 20

The figure taken with permission from Dickinson and Goldstein, (2016) shows the gene tagging strategy with a self-excising selection cassette (SEC). (A) Design of a self-excising cassette for drug selection. SEC contains of a drug resistance gene (hygR), a visual marker [sqt-1(d)], and a heat inducible Cre recombinase (hs::Cre). SEC is flanked by LoxP sites and placed within a synthetic intron with a FP::3xFlag tag, so that the LoxP site that remains after marker excision is within an intron. (B) Illustration of the organization of the his-72 locus and the predicted transcripts from the gene before editing (top), after homologous recombination (middle), and after SEC removal (bottom).

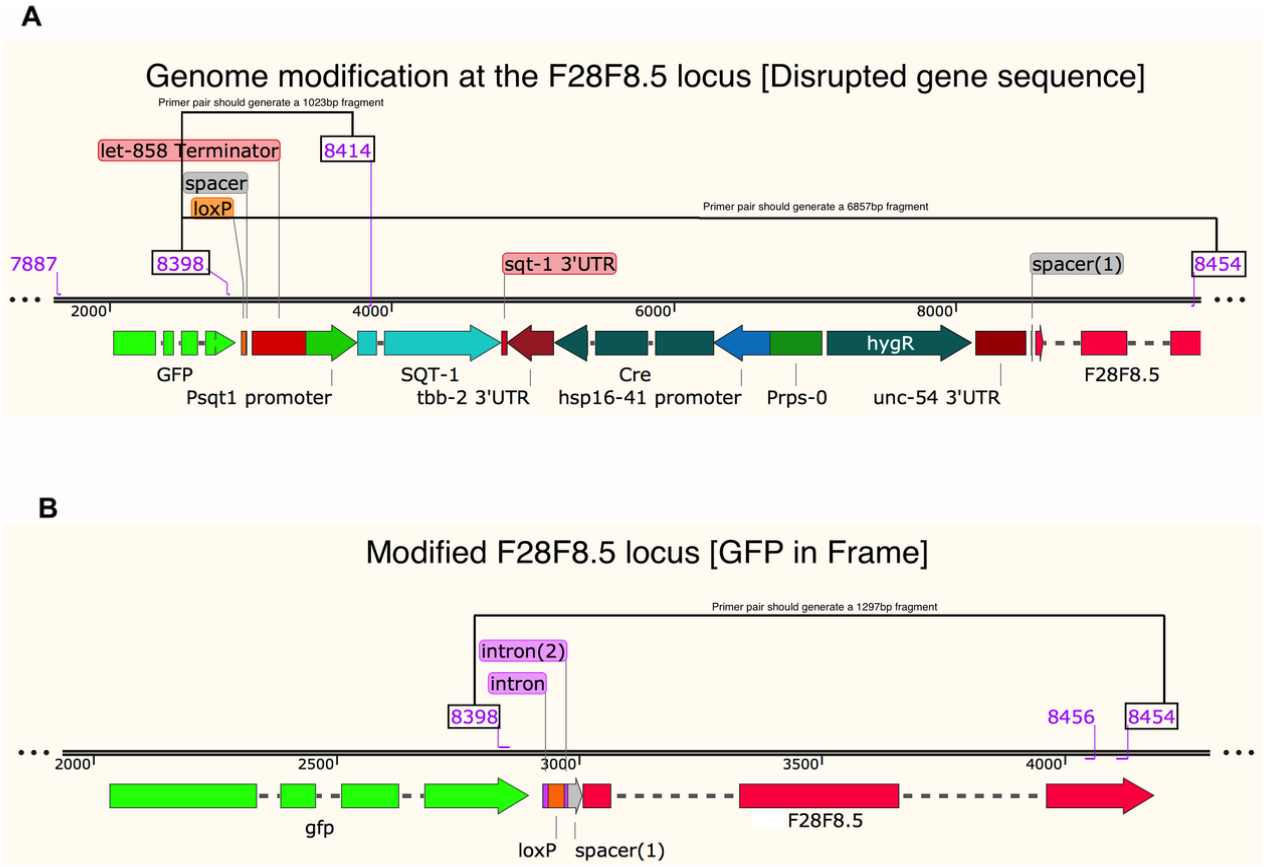


Figure 21

Schematic representation of the two types of genetic modifications created with CRISPR/Cas9 system. A shows the modified natural locus disrupting the F28F8.5 gene sequence. Here the locus is unable to express the mRNA of the F28F8.5 gene, instead, the promoter drives the gfp, thus transcribing free GFP like a transcriptional reporter. B shows the state of the locus after the removal of the SEC induced through heat shock driven by Cre-lox system. After the removal of the SEC, the modified locus is able to natively express F28F8.5 mRNA flagged at the 5′ end with *gfp*.

Sequence analyses

The predicted RXR gene models on JGI database (http://jgi.doe.gov/) (Nordberg et al., 2014) were screened for 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) and the appropriate predicted gene model (protein ID 53515) was selected for further use. The alignments of different RXRs were performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2014) and adjusted/exported as an image file using Jalview (http://www.jalview.org). Protein domain characterization was performed with SMART – a simple modular architecture research tool (Schultz et al., 1998; Letunic et al., 2014). Analysis of HNF4, ERR and COUP-TF were performed similarly. Phylogenetic analysis was done 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. The tree was then visualized using FigTree v1.4.3.

Perilipin orthologues and W01A8.1 sequences were chosen 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 (Remmert et al., 2011; Agarwal et al., 2008) searches as implemented in MPItoolkit (toolkit.tuebingen.mpg.de). Repeat detection used HHrepID module in MPItoolkit. Alignments were displayed and analysed using Jalview app (***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 (Notredame et al., 2000; Di et al., 2011), as well as PROMALS (Pei et al., 2007; Pei et al., 2007a; Pei et al., 2008) and secondary structure predictions performed with PSIPRED (Jones, 1999; Cuff et al., 2000; McGuffin et al., 2000).

Down-regulation of gene expression by RNA interference

Down-regulation of any gene using RNA interference is a well-established technique and was not only initially discovered in *C. elegans* but also functions effectively and is easy to use. Several methods exist for *C. elegans* by which genes can be knocked down (Ahringer, 2006). In our experiments, we used the injection and the feeding method. We kindly request to refer to the original publications for specific details regarding each gene knocked down. The diagram below highlights different ways that the worm can be exposed to dsDNA, furthermore, it also illustrates the basic cellular pathways involved in the interference process. A summary of the mechanism has been published (Grishok, 2005). In the experiments that we conducted, we used either the microinjection or the feeding method. It is believed that the injection method produces the highest knockdown effect. The microinjection method requires a gene-specific dsRNA to be produced using an in vitro method, we did use the commercially available SP6/T7 Riboprobe® *in vitro* Transcription Systems. After dsRNA is made it is directly injected into the *C. elegans* gonads.

The feeding method uses a special strain of *E. coli (HT115(DE3)),* to produce dsRNA inside the bacteria guided by a plasmid encoding the RNA strands. When the worms are feed on the NGM plates layered with the *E. coli* expressing dsRNA, it is absorbed into the organisms spread systemically. Gene *sid-1/ rsd-8* encodes a transmembrane protein that is known to be important for the dsRNA's systemic spread.

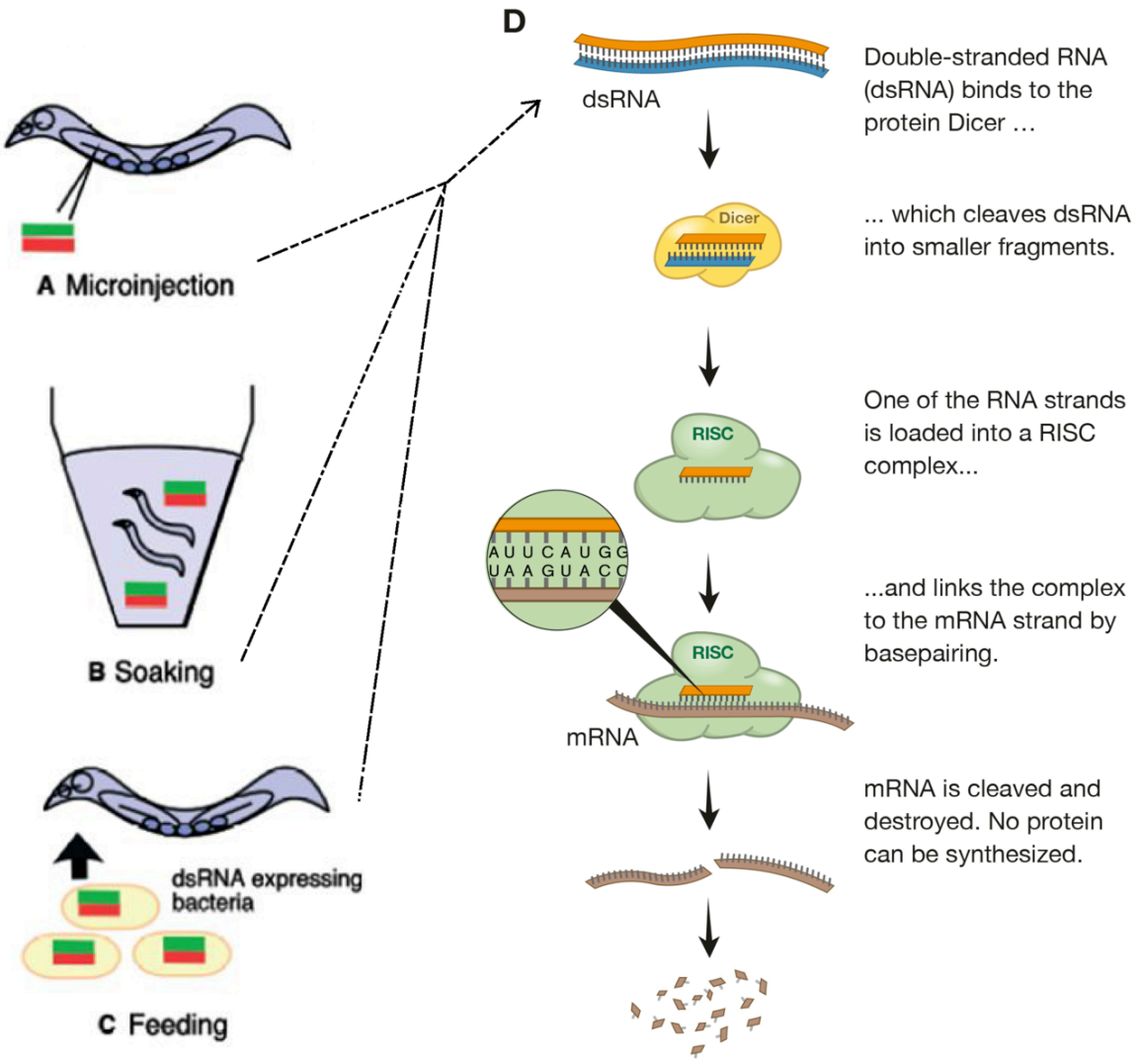


Figure 22

The figure shows the three commonly used techniques to expose *C. elegans* to specific dsRNA that first has to be generated either in vivo (in bacteria) or in vitro (using a transcription system). Once the dsRNA is taken up by the cells it forms complexes with several proteins to form the RISC complex (RNA-induced silencing complex). Image taken and modified from MLA style: Press release. NobelPrize.org. Nobel Media AB 2019. Fri. 22 Feb 2019. <***https://www.nobelprize.org/prizes/medicine/2006/press-release/***>.

Fecundity and brood size assay

Fecundity measurement following RNAi (injection method) was performed using a total of 50 young adult worms (25 control and 25 inhibited by RNAi specific for W01A8.1). Progeny was counted 24 hours and at 48 hours after injections. Brood size assay was performed for W01A8.1 disrupted animals and controls (n = 15 for each group). The progeny was determined over 6 days. The experiments were conducted at room temperature of ~22 °C.

Fecundity measurement after RNAi using feeding protocol was performed over two generations to maximize the knockdown effect. For this, a semi-synchronized population was isolated using standard WormBook bleaching protocol (***http://www.wormbook.org/***). Hatched L1 stage worms were

then placed on NGM agar RNAi (W01A8.1 specific and control) plates at ~22 °C. Small, synchronized populations, of parents (P0) were transferred to fresh RNAi plates and allowed to lay progeny (F1). F1 generation animals were transferred to new RNAi plates and F2 generation was scored for a total of 21 F1 parents in each group. The experiments were repeated twice to confirm the results.

RNA isolation and cDNA synthesis

For RNA extraction from *C. elegans*, cultured nematodes were washed and collected in water. After which they were pelleted by brief centrifugation at 200 *g*. Depending on the exact experiment, either the pellet was frozen at -80°C after removal of excess water or directly used for RNA extraction. In both cases the pellet was re-suspended in re-suspension buffer (0.5% SDS; 5% 2-mercaptoethanol; 10mM EDTA; 10mM Tris/HCl (pH 7.5) with proteinase K, gently mixed for 1 min and incubated for ca. 20 minutes at 55°C (the time and volume varied depending on the number of the worms in each pallet). After lysis TRIzol® Reagent (Invitrogen) was added to the mixture and the standard manufacturer's protocol was followed to obtain total RNA. Samples were then treated with DNase I for ca. 30 min at 37°C and again TRIzol-chloroform or phenol-chloroform purified and finally precipitated with ethanol the to obtain DNA free total RNA in DEPC water.

For *Trichoplax* RNA extraction, several animals were removed and added to TRIZol reagent (Invitrogen) and simply mixed until dissolved. After which the standard manufacturer's protocol was used for cDNA synthesis (same as mentioned above for *C. elegans*).

Human total RNA was also extracted using TRIzol® Reagent (Invitrogen) from peripheral blood lymphocytes and fat tissue under standard manufacturer guidance.

Complementary DNA (cDNA) was prepared with SuperScript® III First-Strand Synthesis System (Invitrogen) using random hexamers and/or oligo(dT) priming based on the standard manufacturer guidance.

Single or multiple worm DNA analysis

Single animals were placed into 5 µl of worm lysis buffer (10mM Tris–HCl pH 8.3, 50mM KCl, 2.5mM MgCl2, 0.45% NP-40, 0.45% Tween 20, 0.01% Gelatin and 500µg/ml fresh proteinase K) in a PCR tube. The tube was then frozen at -70°C to -80 °C for 5 min (or longer) before placing the tube into a thermal cycler and run under the following conditions: heated to 60°C for 60min to allow for worm lysis. Then the proteinase K is inactivated by heating to 95°C for 15-20 mins. Post-lysis, the samples were either directly used or stored for later analysis by PCR. For the PCR, a reaction mix (45µl) targeting the template of choice was added and cycled for ~35 times with Q5® Hot Start DNA polymerase (New England Biolabs). Similarly, genomic DNA was prepared from selected nematode culture plates and used for further screening by PCR and sequencing.

Transcript quantification

For quantitative RT-PCR, the technique described by Ly, Reid, and Snell (2015) was used with modifications. For the determination of W01A8.1 and F28F8.5 transcripts we used the Roche Universal Probe Library technique (Hoffmann-La Roche, Basel, Switzerland). Universal probe library and primers designed with the help of ProbeFinder Assay Design Software were used and qPCR was run on LightCycler 2.0 purchased from Roche (Roche, s.r.o. Prague, Czech Republic). 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 of *W01A8.1* and *F28F8.5* was normalized to *ama-1.*

The Droplet digital PCR based measurements were performed on a QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). ddPCR was used for accurate quantification of small amount RNA obtained from cultured *T. adhaerens.* For each set of experiments about 4 to 10 animals were used.

The quantification was used to determine the effectiveness of RNAi (for *W01A8.1* gene) as well as to determine the true loss of *F28F8.5* gene expression. For the quantification of the expression of *F28F8.5* in homozygous mutants compared to N2 wild-type we a more efficient method to reduce RNA and cDNA loss as the starting samples were very little. Five adult homozygous animals with the edited disrupted *F28F8.5* gene, recognised by the phenotype, and the same number of WT hermaphrodites with a minimum number of formed embryos were manually harvested and collected in separate Eppendorf tubes. The reverse transcription from these samples was done using the Maxima H Minus cDNA synthesis kit (Thermo Fischer, Waltham, MA, USA) as recommended by the manufacturer.

The details of each of the experiments are thoroughly provided in each respective publication.

LipidTox staining

The lipid staining protocol was performed according to O'Rourke et al. (2009) with modifications. Approximately 200 – 500 animals were harvested from NGM plates with 1X PBS and washed several times to remove bacteria and pelleted at 1,500 *g*. To the pellet, 500µl 2X MRWB buffer (40mM NaCl, 14mM Na_2EGTA, 160mM KCl, 0.4mM Spermine, 1mM Spermidine 3HCl, 30mM NaPIPES pH 7.4, 0.2% beta-ME) and 100µl of 20% paraformaldehyde were added and the volume was adjusted up to 1ml with 1X PBS. The tubes were inverted several times to mix the worms in solution after which they were allowed to fix for about an hour at room temperature with gentle shaking.

Animals, after fixation, were then pelleted at 1,500 *g* and washed 3 times with 1ml Tris–HCl buffer (100mM, pH 7.4). After the third wash, the supernatant was discarded with a remaining volume of 100µl to which 650µl of Tris-HCL buffer followed by 250µl of fresh/frozen reduction buffer (100mM Tris-Cl pH 7.4, 40mM DTT) was added. Worms were then left to shake for about 30mins at room temperature. Worms were washed 3 times in 1X PBS after reduction. After the final PBS wash, the volume was brought up to 0.5ml and then 0.5ml of LipidTox (Red) (1:500 dilution) (Invitrogen) was

added making a final volume of 1ml and concentration of 1:1000 of LipidTox. The worms were left in the dark for at least an hour shaking before viewing.

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).

Microscopy and Imaging

Fluorescence microscopy and Nomarski optics microscopy were done using an Olympus BX60 microscope equipped with DP30BW CD camera (Olympus, Tokyo, Japan). For live imaging the worms were anaesthetized with levamisole and placed on a cover glass with a thin layer of 2% agarose.

Coherent Anti-Stokes Raman Scattering Microscopy (CARS)

The following diagram represents the working principle of the CARS microscopy used for lipid analysis in living cells as published by Potcoava et al., (2014). We utilised the method to analyse fat content in living animals. The CARS systems allow visualisation of lipids of specific categories by tuning into symmetric CH_2 vibrations of specific fat composing molecules (Zumbusch et al., 2013).

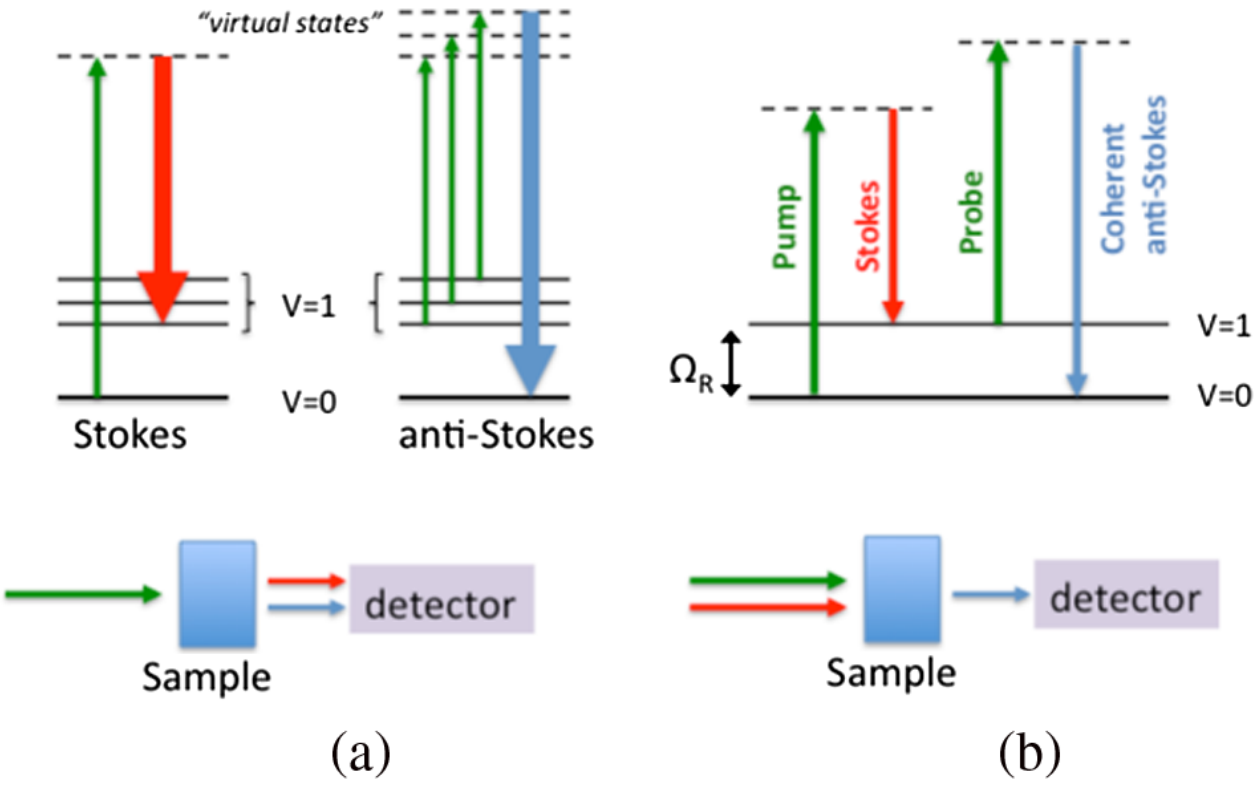


Figure 23

The Principle of Raman scattering mechanisms of (a) spontaneous Raman and (b) narrowband CARS shown by Jablonski diagram (energy level diagram). Arrows represent photons (longer length denotes higher photon energy), V is the vibrational level, ΩR is the resonant vibrational frequency of a given vibrational mode. For CARS, the laser source consists of two time-locked pulse trains of different wavelengths (pump + stokes) such that the difference in photon energy corresponds to the vibrational mode to be imaged. The anti-Stokes photon is detected indicating the strength of the signal at a given vibrational mode. Image used from Potcoava et al., (2014) under Creative Commons Attribution 3.0 Unported License.

The CARS images were acquired with a Leica TCS SP8 CARS system (Leica Microsystems, Mannheim, Germany) consisting of a TCS SP8 confocal microscope combined with a picoEmerald laser (APE, Berlin, Germany) offering a fixed Stokes laser line of 1064.5nm and a tuneable Pump line from an optical parametric oscillator (780nm to 940nm). A HC PL IRAPO 40x water immersion objective was used for the imaging and CARS signal was detected with a non-descanned photon multiplier tube detector at the transmitted light side. For imaging of CH_2 vibration with a Raman shift of 2,868cm−1 pump wavelength of 815.5nm was used. The CARS signal was selected with a CARS2000 filter cube placed in front of the detector. Adult animals and embryos were completely scanned and recorded as stacks of focal planes. Recordings for quantitative analysis were done at fixed settings for mutant and wild type.

Image analysis

Single focal planes, containing the highest number of CARS positive structures, from stacks of representative embryos and adult hermaphrodites were selected and analysed using ImageJ computer program (***http://imagej.nih.gov/ij/***). Analysis of embryos was performed on images of seven mutant and seven wild type embryos inside gravid hermaphrodites (only one and two cell early embryonic stages were chosen for comparison). Analysis of adult somatic tissue was performed on the distal body region and we compared five different mutant adults with five different wild type adults. Automatic particle counting feature of ImageJ program was used for determinating the number and the area of CARS positive structures with manual thresholding as described on ***http://imagej.net/Particle_Analysis***. Image area required for analysis was first selected (the area outside the selected zone was cleared) then the image was converted to an 8-bit scale. A manual threshold was applied with settings yielding the biggest number of individually recognisable structures (adult tissue threshold setting range was 36–200 and for embryos 11–13 to 200). Overlapping structures were separated using the 'Watershed' command and also by the manual line draw feature. 'Analyse particles' command generated data sets containing the number and area of particles. Microsoft Excel 2003 was used to perform statistical analysis and two-tailed Student's t-test for determining the p-value. Raw data sets are provided as supplement and labels in the Excel tables correspond to the marked images also provided as supplement.

Confocal and Fluorescence-lifetime imaging microscopy

Confocal microscopy of live homozygous animals was performed using an inverted Leica SP8 TCS SMD FLIM system equipped with a 63× 1.2 NA water immersion objective, a pulsed white light laser (470–670 nm), AOBS and two internal hybrid single photon counting detectors, and operated by Leica Application Suite X program (Leica Microsystems, Wetzlar, Germany). The GFP fluorescence was excited at a wavelength of 488 nm and the emitted light was simultaneously recorded in two spectral ranges (Channel 1—495 nm to 525 nm, Channel 2—525 nm to 580 nm; the two-channel setup was used to help resolve between spectrally different autofluorescence and GFP fluorescence signals).

Each fluorescent molecule has its own life in an excited state. By detecting differences in lifetime, it is possible to distinguish between various molecules present in a given sample. Furthermore, the technique can be employed to identify autofluorescence. Excited missions from fluorescent proteins like GFP tend to decay slowly compared to autofluorescence produced by other complements of the cells.

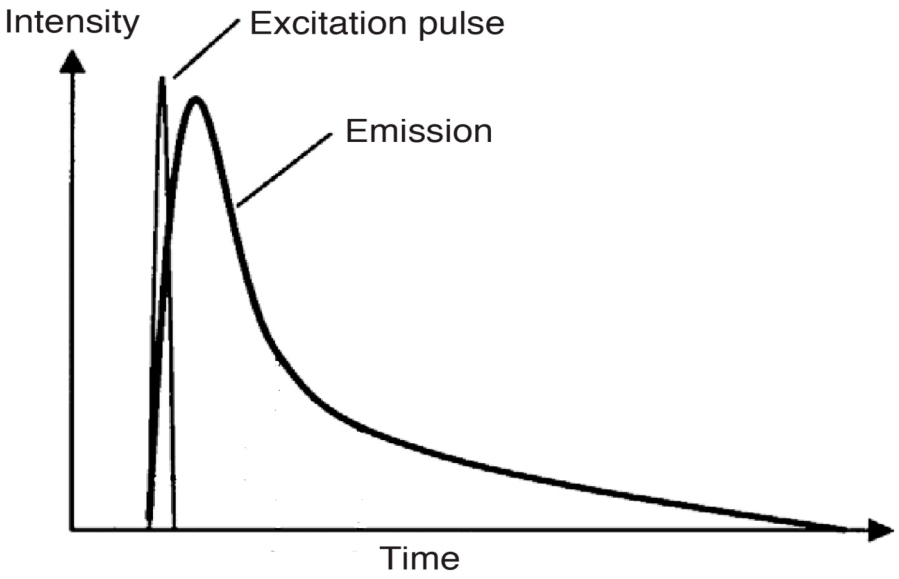


Figure 24

The basic concept of fluorescence lifetime imaging microscopy is that following pulsed excitation (from a light source such as a femto-second pulsed laser), the sample emission intensity decays exponentially. Time-gated FLIM captures this decay curve by detecting the signals in a few gates and use the signals to fit the curve and calculate the decay characteristic time constant as the fluorescence lifetime. Figure adopted with permission from Chang et al., (2007).

For FLIM acquisitions the single photon counting signal from the internal hybrid detectors, acquired during confocal acquisitions, was simultaneously processed by HydraHarp400 TCSPC electronics (PicoQuant, Berlin, Germany) and information about the arrival times of all photons was stored to a hard-drive in TTTR data format. TTTR is freely accessible at ***https://www.picoquant.com/images/uploads/page/files/14528/technote_tttr.pdf***. 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*. The signal from both time-synchronized channels was added up. The false colour scale was used and is based on the average photon arrival time, with blue colour representing short lifetime and red colour long lifetime fluorescence (image found in original article).

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.

Bl21 pLysS bacteria were transformed with RXR cloned cDNA inserted into pGEX-2T vector (Amersham Pharmacia Biotech, Amsterdam, UK). Stocks of transformed bacteria were stored in 8% glycerol according to the Novagen pET System Manual for protein expression. Bacteria were then scraped from stock and incubated in Liquid Broth (LB) with ampicillin (100µg/ml) and chloramphenicol (34µg/ml) overnight. The culture was then used to inoculate a 100ml of LB with antibiotics and grown to an optical density of OD_{600} = 0.6 to 0.8 at 37°C. After this the culture was induced with 100µl 1M IPTG (isopropyl-D-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO, USA) and moved to 25°C (RT) for 5 hours. The culture was then centrifuged at 9,000 *g* for 15 minutes, the supernatant discarded and the bacterial pellet was resuspended in 10 ml GST binding buffer (25mM Tris pH 7.5, 150mM NaCl, 1mM EDTA) with the addition of protease inhibitor (S8820 Sigma Fast, Sigma-Aldrich, St. Louis, MO, USA or cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche, Basel, Switzerland). Bacteria were then lysed by 6 × 20 s ultrasonication on ice (50 watts, 30 kHz, highest setting—100%) (Ultrasonic Processor UP50H, Hielscher Ultrasonics GmbH, Teltow, Germany) and subsequently incubated with 15 to 20mg glutathione agarose beads (Sigma-Aldrich®) prepared according to manufacturer's protocol. Incubation took place at 4°C for about 10 hours after which the beads were washed according to instructions, resuspended in regeneration buffer (50mM Tris–HCl pH7.4, 120mM KCl, 1mM EDTA, 8% glycerol (v/v), 5mM DTT) or 50mM TRIS–HCl pH 7.4 + 9% (v/v) glycerol for subsequent thrombin (bovine plasma, Sigma-Aldrich®) cleavage, if performed, and then adjusted for regeneration buffer conditions. GST-TaRXR was eluted from glutathione agarose beads using 10mM reduced glutathione (Sigma-Aldrich, StLouis, Mo, USA) in 50mM Tris–HCl buffer pH 8.0. The size of the GST-TaRXR fusion protein was checked by polyacrylamide gel electrophoresis. Thrombin cleavage was carried out at room temperatire for 4 hours and the quality of the purified protein was assessed by SDS-PAGE.

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 Escherichia coli cells and the production of protein was induced by isopropyl β-d-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich). The rest of the protocol was same as used above to produce GST-TaRXR.

The MDT-6 protein synthesis was performed by cloning the coding region of *mdt-6* into pTNT vector (Promega, Madison, WI, USA) and expressed in the rabbit reticulocyte TNT-system (Promega, Madison, WI, USA) based on the standard manufacture's guidance. The in vitro transcribed protein was labelled using ^{35}S Methionine (Institute of Isotopes, Budapest, Hungary).

The Mediator subunit MDT-30's coding sequence was cloned into pET28a(+) vector (Addgene, Cambridge, MA, USA) and transformed into BL21 *E. coli* cells and induced by IPTG. The lysate from bacteria producing His6-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 labelled Mediator subunits were incubated with glutathione–agarose (Sigma-Aldrich, St. Louis, MO, USA) adsorbed with equal amounts of GST or GST-F28F8.5.

Radioactive retinoic acid binding assay

Radioactive ^{3}H-labelled 9-*cis*-RA and ^{3}H-labelled AT-RA were obtained from PerkinElmer (Waltham, MA, USA). Binding was performed in 100 µl binding buffer (50mM Tris–HCl pH7.4, 120mM KCl, 1mM EDTA, 5mM DTT, 8% glycerol (v/v), 0.3% to 0.5% (w/v) CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, Sigma-Aldrich)) for 2 hours on wet ice in a dark room. Work with retinoids was always carried out under indirect illumination with a 60W, 120V yellow light bulb (BugLite, General Electric Co, Nela Parc, Cleveland Oh, USA) as described Cahnmann, (1995). The protein used for binding was either GST-RXR fusion protein on beads with about 375ng/assay and thrombin-cleaved RXR. For estimation of specific binding, 200-fold-excess of either 9-*cis*-RA or AT-RA (Sigma-Aldrich) was used. In case of GST-RXR fusion protein, 50 µl of the supernatant was removed after 30 sec at 1300 *g* and washed 3x with 1000µl wash buffer (50mM Tris–HCl pH7.4, 120mMKCl, 1mM EDTA, 5mM DTT, 8% (v/v) glycerol, 0.5% (w/v) CHAPS) removing 900µl after each wash. For cleaved RXR protein, 10µl hydroxyapatite slurry (AG-1 XB Resin, Bio-Rad, Hercules, CA, USA) suspended in binding buffer (12.7mg/100µl) were added to the assay and mixed twice, collecting the apatite slurry by centrifugation (15s at 600 *g*). 95µl of the supernatant was removed and the slurry washed twice with 1ml of wash buffer, removing 900µl after each wash. The radioactivity of the GST-fusion protein and cleaved protein was measured using Packard Tri-Carb 1600TR Liquid Scintillation Analyzer (Packard, A Canberra Company, Canberra Industries, Meriden, CT, USA) and Ultima Gold Scintillation Fluid (PerkinElmer, Waltham, MA, USA). 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. As mentioned before, ^{35}S Methionine labelled MDT-6 was translated using the rabbit reticulocyte TNT-system (Promega). 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 (Sigma-Aldrich) 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 visualized 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 (monoclonal anti-FLAG, M2 (Sigma-Aldrich) and quantified densitometrically by ImageJ computer program (https://imagej.nih.gov/ij/download.html).

Results

The results presented have been divided into three parts, each part corresponds to an article published by myself in collaboration my colleagues.

Results of Part I

The following section was reprinted and modified under the Creative Commons CC-BY 4.0 license from Novotný et al. (2017). Only the most relevant parts, contributing to the philosophy of this dissertation, have been shown here.

Trichoplax adhaerens retinoid X receptor is highly conserved

Searching the JGI's (Joint Genome Institute of the U.S. Department of Energy) *Trichoplax* genome database allowed us to identify a RXR orthologue as well as verify the full length transcript containing the DBD and LBD. The sequence was previously not annotated as the 'best model'. Protein Blast analysis showed a high sequence similarity to mammalian RXR (human and mouse) with 66% overall sequence identity to human RXR alpha.

SMART (a simple modular architecture research tool) analysis of the proposed RXR sequence in *Trichoplax* showed a zinc finger DNA binding domain (amino acid residues 16–87) and a ligand binding domain (amino acid residues 155–342) 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. As shown below (Fig. 25) the heptad repeat LLLRLPAL, proposed for dimerization activity (Forman and Samuels, 1990; Forman and Samuels, 1990a), 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) were present. From the eleven AA residues known to be critical for 9-cis-RA binding (A271, A272, Q 275, L 309, F 313, R 316, L 326, A 327, R 371, C 432, H 435) nine are conserved and the remaining two amino acids are substituted (A327S, and C432T (C432A in *Tripedalia cystophora*)). This data showing a high sequence identity allowed us to hypothesise that the RXR in *Trichoplax* has similar, if not same, 9-*cis*-retinoic acid binding capability as well as DNA binding capability.

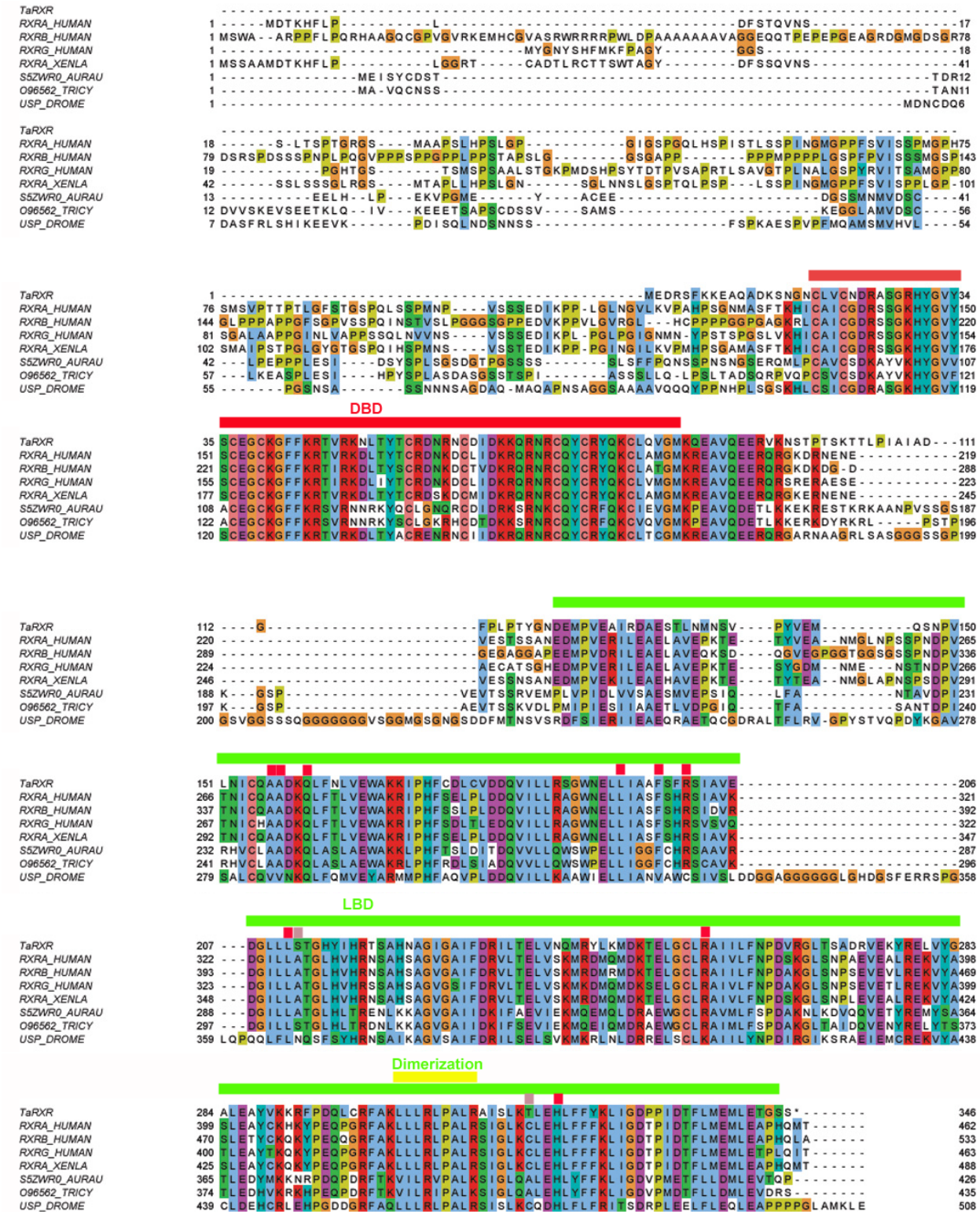


Figure 25

Multiple sequence alignment of selected metazoan homologues of RXR compared with Trichoplax RXR. Sequences were aligned with ClustalO, AA residue types coloured according to Clustal scheme in Jalview. Sequences from top to bottom (organism, identifier): *Trichoplax adhaerens*, TaRXR ID 53515; *Homo sapiens*, sp|P19793|RXRA_HUMAN; *Homo sapiens*, sp|P28702|RXRB_HUMAN; *Homo sapiens*, sp|P48443|RXRG_HUMAN; *Xenopus laevis*, RXR alpha, sp|P51128|RXRA_XENLA; *Aurelia aurita*, RXR, tr|S5ZWR0|S5ZWR0_AURAU Retinoid X receptor; *Tripedalia cystophora*, RXR, tr|O96562|O96562_TRICY

Retinoic X receptor; *Drosophila melanogaster*, USP, sp|P20153|USP_DROME. DNA binding domain (DBD, red line), Ligand binding domain (LBD, green line), dimerization domain (yellow line) and amino acid residues critical for 9-*cis*-RA binding (conserved–red rectangles, not conserved–pink rectangles) are indicated. To allow for specific colour preferences one may download the compared sequences (downloadable from the original article's supplementary data) and create the Clustal scheme with different colour specifications using the Jalview program (***http://www.jalview.org/***).

RXR of *Trichoplax adhaerens* has conserved ligand binding ability

To analyse the binding properties of RXR in *Trichoplax*, we expressed RXR from *Trichoplax* as a GST-fusion protein (GST-TaRXR) in bacteria. 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 (Fig. 26A and B) 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 (Fig. 26C). However, all-*trans*-retinoic acid was unable to show high affinity binding to GST-TaRXR.

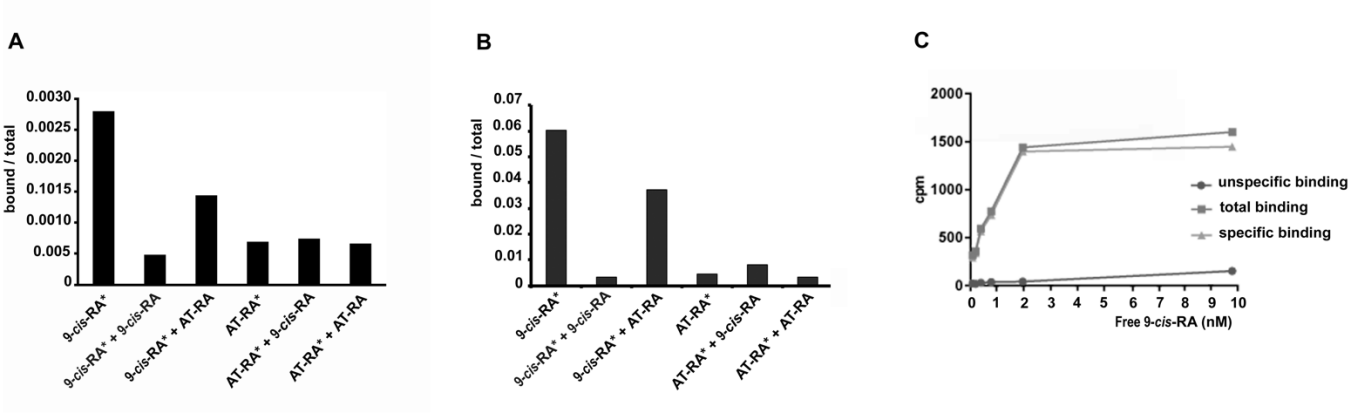


Figure 26

In vitro binding of retinoic acids to RXR from *T. adhaerens*. A) Single point analysis of binding preference of *T. adhaerens* RXR (thrombin cleaved) to ^{3}H-labelled 9-*cis*-RA over all-*trans*-RA. Radioactive 9-*cis*-RA (9-*cis*-RA∗) binds at a concentration of 4nM to 200 nanograms of *T. adhaerens* RXR. 200-fold excess of unlabeled 9-cis-RA displaces more than 80% of labelled 9-cis-RA from binding to *T. adhaerens* RXR (9-cis-RA∗ + 9-cis-RA) while the same molar excess of all-*trans*-RA (9-cis-RA∗ + AT-RA) which is likely to contain approximately 1% spontaneously isomerized 9-*cis*- RA, competes away less than 50% of bound ^{3}H-labeled 9-*cis*-RA. Radioactive ^{3}H-labeld all-*trans*-RA (AT-RA∗) at identical conditions binds only slightly more than the observed non-specific binding. This interaction is not displaced by the excess of non-labelled 9-cis-RA (AT-RA∗ + 9-cis-RA) nor non-labelled all-trans-RA (AT-RA∗ + AT-RA). Results are expressed as a ratio of the radioactivity bound to TaRXR/total radioactivity used for the binding at the given condition. (B) Analysis of binding properties of *T. adhaerens* RXR (in the form of GST-TaRXR) to ^{3}H-labelled 9-*cis*-RA and ^{3}H-labelled all-*trans*-RA. The experiment differs from the experiment shown in A in 5-fold greater amount of radioactive all-*trans*-RA (and therefore only 40-fold excess of non-radioactive competitors). The experiment shows

identical binding properties of GST-TaRXR as those observed with thrombin cleaved TaRXR. (C) Kinetic analysis of binding of ^{3}H-labelled 9-*cis*-RA to *T. adhaerens* RXR prepared as GST-fusion protein (GST-TaRXR). The plateau is reached at around 3 to 5 × 10^{-9} M.

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

To show whether 9-*cis*-RA has an observable biological effect on *T. adharens in vivo*. As a known metabolic regulator in many other species we speculated that TaRXR is likely to be involved in the regulation of metabolic events. RXR is a dimerization partner of TR in mammals and together these two NRs are regulators of a wide range of metabolic pathways. We, therefore, searched for an orthologue of vertebrate L-malate-NADP^{+} oxidoreductase (EC 1.1.1.40) in *T. adhaerens* genome since this enzyme is an established reporter of the state of thyroid hormone dependent regulation (Dozin et al., 1985, Dozin et al., 1985a, Petty et al., 1990).

The sequence of the *T. adhaerens* likely orthologue of vertebrate L-malate-NADP^{+} oxidoreductase was retrieved from the *Trichoplax* genomic database together with its presumed promoter based on the predicted sequence.

A highly sensitive method of quantitative PCR (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.

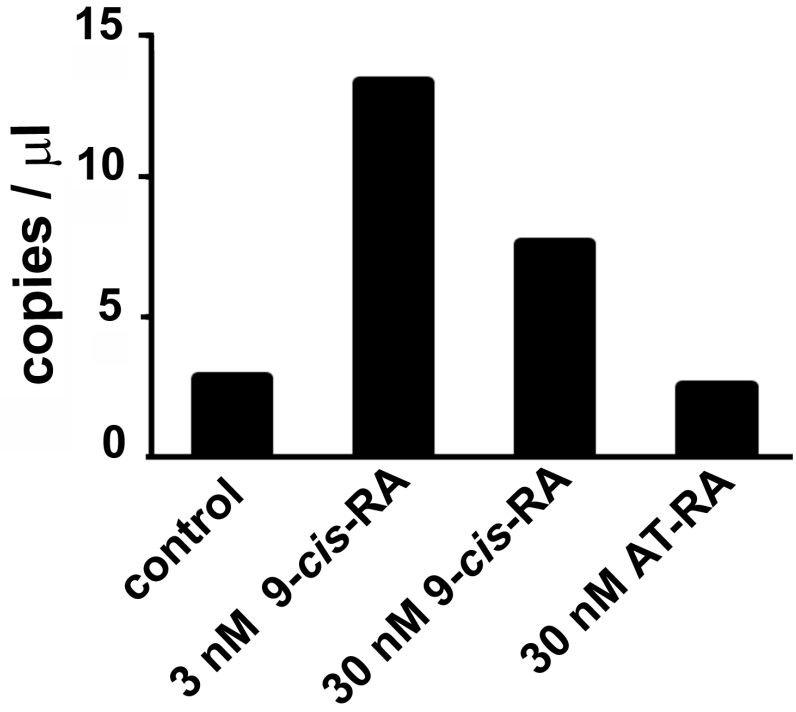


Figure 27

The effect of 9-*cis*-RA on the expression of the *T. adhaerens* closest putative homologue and likely orthologue of L-malate-NADP+ oxidoreductase (EC1.1.1.40). Ten to fifteen animals were cultured in the dark overnight with indicated ligands or in medium containing only the solvent used for ligand solutions. Total RNA and cDNA were prepared using identical conditions and diluted to the same working concentration suitable for ddPCR. In repeated experiments, incubation with 3nM 9-*cis*-RA induced expression of the putative *T. adhaerens* L-malate-NADP+ oxidoreductase more than four times. Incubation with 30nM 9-*cis*-RA induced enzyme expression also, but to a lesser extent and 30nM all-*trans*-RA (AT-RA) did not upregulate the expression of the predicted L-malate-NADP+ oxidoreductase.

Results of Part II

The following section was reprinted and modified under the Creative Commons CC-BY 4.0 license from Chughtai et al., (2015). The most relevant parts (including some supplementary data) have been shown here.

W01A8.1 shows structural similarities to vertebrate perilipins

The *mdt-28* gene in the *C. elegans* database (*W01A8.1)* was known to code for three possible isoforms of the protein MDT-28, these were labelled as isofrom a, b and c (Fig.28). Each of the isofrom of the protein was much larger in terms of sequence and mass compared to its putative orthologues in mammals. The human MED28 is only 178 amino acid long, as opposed to W01A8.1, who's smallest isofrom (b) is actually 385 amino acids long. This extra length was already a surprising fact for us and certainly made W01A8.1 an interesting target of the investigation.

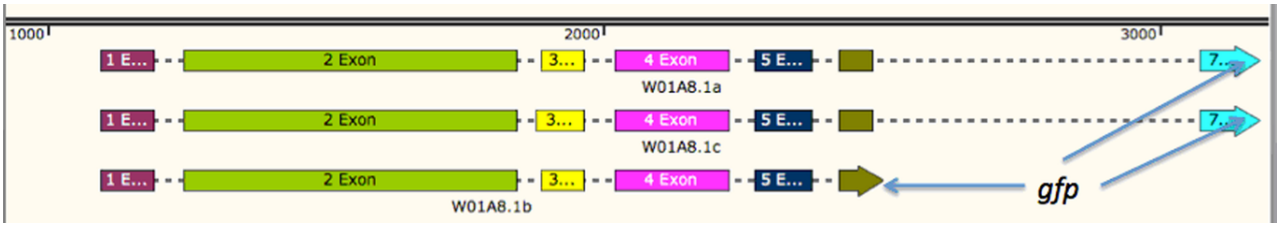


Figure 28

This is a scheme of W01A8.1 gene, expressed isoforms and preparation of gfp tagged transgene by SOEing PCR. Three different protein isoforms are expressed from the W01A8.1 gene. The gene and the expressed proteins are denominated as W01A8.1 a, b and c (as accessed in Wormbase WS 246 on March 14, 2015). The gene coding for GFP is inserted before the STOP codon in the 7th exon (marked as W01A8.1a/c::gfp) or in the 6th exon of isoform b (marked as W01A8.1b::gfp). The transgene based on the first construct (covering the upper two isoforms a and c) may lead to the expression of both isoforms a or c fused to GFP dependent on the cellular context and is likely to lead also to overexpression of untagged isoform b from the extra-chromosomal array. The genomic map was designed using SnapGene software (from GSL Biotech; available at snapgene.com).

In UniProt Knowledgebase human mediator complex 28 (Q9H204) was submitted for BLASTp, PSI-BLAST and DELTA-BLAST to find possible alignments. Unfortunately, this resulted in no significant matches. We then tried a reverse bioinformatic approach and submitted W01A8.1 as a query

in BLASTp, PSI-BLAST and DELTA-BLAST. This however also resulted in no targets with a significant score. When running Conserved Domain search by submitting W01A8.1a sequence as a query we were able to identify two different domains: a MED28 belonging to MED28 superfamily domain a Perilipin domain belonging to the Perilipin superfamily domain.

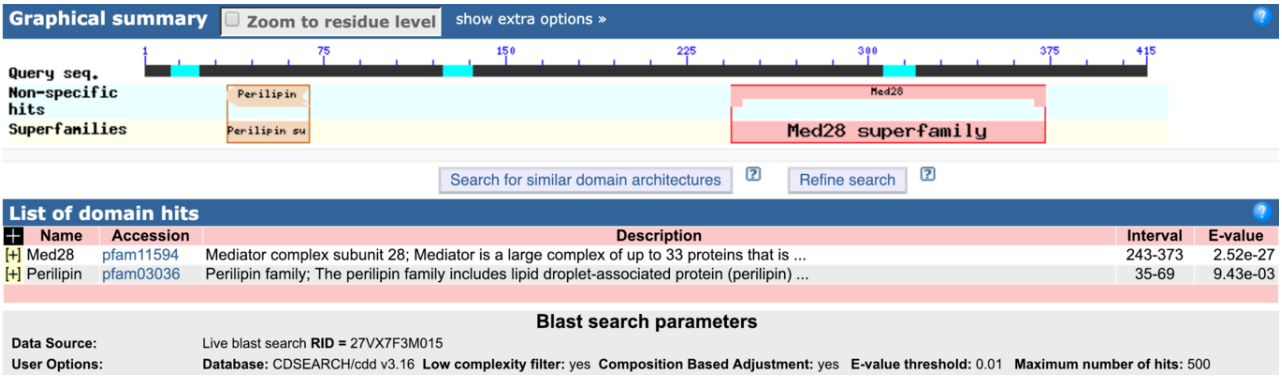


Figure 29

Conserved Domain Database search excerpt with W01A8.1a (Wormbase sequence) as submitted query. The results show two possible domains one belonging to the Perilipin superfamily and the other to the MED28 superfamily.

Despite these non-specific results when a sequence alignment of chordate perilipins 2 and 3 (OMA database) was submitted as a query in PSI-BLAST, the *C. elegans* protein W01A8.1a (Q23095_CAEEL) was identified as a highly significant hit ($E = 3 \times 10^{-13}$). A reciprocal PSI-BLAST search with the aligned closest nematode homologues of W01A8.1a identified chordate perilipins as strong hits with human Perilipin 2 (significance score $E = 10^{-53}$) appearing in the second iteration of the search. Similarly, HHpred profile-to-profile searches with human Perilipin sequences as a query of the *C. elegans* proteome identified proteins coded by W01A8.1 (a, b or c) and reciprocally W01A8.1a showed profile homology to all human Perilipins and the corresponding Pfam (Punta et al., 2012) Perilipin profile (PF03036). Each available nematode proteome contained only a single such Perilipin-related sequence, in stark contrast to the insect and chordate proteomes that had 2–5 Perilipin paralogues. 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.

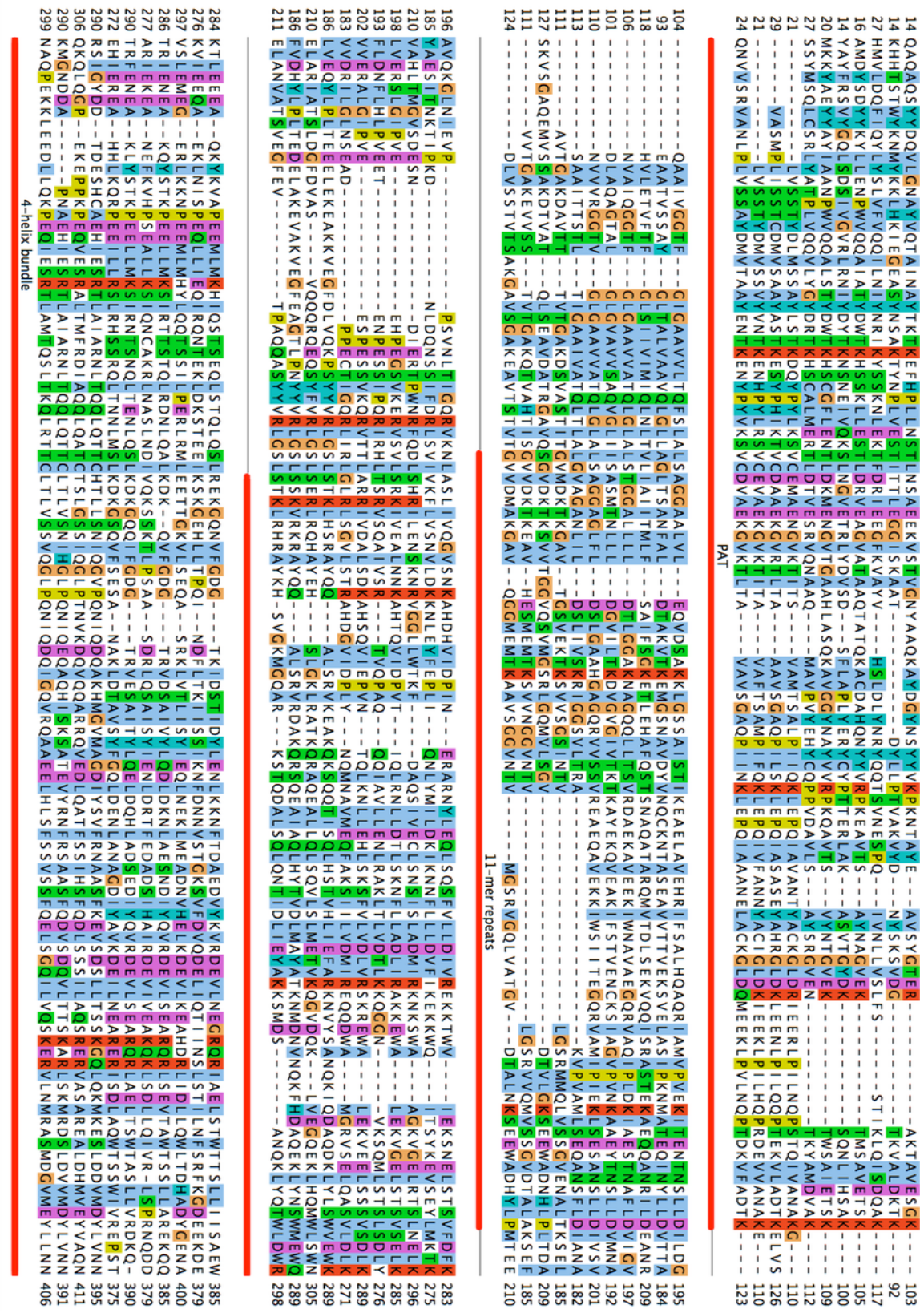


Figure 30

Identification of *C. elegans* protein W01A8.1a as a close orthologue of vertebrate Perilipin. *C. elegans* protein W01A8.1a is compared with nematode orthologues of pairwise sequence identity lower than 70%

and with Plin2 and Plin3 from two diverse vertebrates. Three Perilipin specific domains can be identified (marked as red) were identified through homology with human Plin3. The six 11-mer repeats in the W01A8.1a were established using the HHrepID algorithm (Biegert and Söding, 2008). The N-Terminal PAT domain is through to interact with hormone-sensitive lipase (HSL). The central domain consisting the imperfect 11-mer repeats forming amphipathic helices and the apolipoprotein-like 4-helix bundle is known to be responsible for the LD affinity and the later has been reported to interact with ABDH5 in mammalian Plin1 and 3 (Brasaemle, 2007). Alignment was done using T-coffee alignment of all available nematode sequences aligned with vertebrate Plin2 and 3 sequences in three iterations using ProfileAlign routine in MyHits suite (myhits.isb-sib.ch). Selected sequences from top to bottom: (Species, database identifier): *Caenorhabditis elegans*, Q23095; *Strongyloides ratti*, CACX01001972.1; *Loa loa*, E1G5Y0 and ADBU02007219.1; *Haemonchus contortus*, CDJ80228.1; *Bursaphelenchus xylophilus*, CADV01008520.1; *Heterorhabditis bacteriophora*, ES742365.1 and ACKM01001830.1; *Ascaris suum*, U1NU60; *Homo sapiens* 2, PLIN2_HUMAN; *Homo sapiens* 3, PLIN3_HUMAN; *Latimeria chalumnae 2*, H3AYC0; *Latimeria chalumnae 3*, GAAA01019375.1. Nucleotide sequences were translated with Wise2 program (Birney et al., 2004). Amino acid types are coloured according to the Clustal scheme (***jalview.org/help/html/colourSchemes/clustal.html***).

The 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) covering approximately amino acids 10–100, 125–190 and 220–380 respectively in W01A8.1a. 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.

Protein databases annotated W01A8.1 as Mediator Complex subunit 28, hence the official protein name assignment of MDT-28 in WormBase (WS246). Pfam database (Punta et al., 2012) based the Mediator 28 Hidden Markov model profile on a seed alignment of bovine and mosquito Mediator 28 sequences with W01A8.1. This very profile was probably used subsequently in all automatic annotations of the nematode sequences. However, no substantial homology between W01A8.1 and Mediator 28 exists as shown in the above searches. Since using the WormBase name of W01A8.1 (MDT-28) would be misleading, the gene is referred here by the cosmid name *W01A8.1*, which gives rise to at least three protein isoforms designated W01A8.1a, W01A8.1b, and W01A8.1c from at least seven different transcripts (*W01A8.1a.1*, *W01A8.1a.2*, *W01A8.1b.1*, *W01A8.1b.2*, *W01A8.1b.3*, *W01A8.1c.1*, *W01A8.1c.2*). The three protein isoforms are 415, 385, and 418 amino acid residues in length for isoform a, b, and c, respectively. According to the *C. elegans* nomenclature, we suggest to rename *W01A8.1* as *plin-1* (isoform a, b, and c) and proteins PLIN-1 (isoform a, b, and c).

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

If the proteins encoded by W01A8.1 act as perilipins, they would be expected to be associated with lipid droplets (Kozusko et al., 2015). To test this, we created translational reporter transgenes

regulated by the putative endogenous promoter expressing isoform b and lines in which the genomic locus was tagged by an in-frame C-terminal GFP cassette (Fig. 28). The second transgene, W01A8.1a/c::gfp, is likely to express not only high levels of a and c tagged isoforms, but also the native isoform b. 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 (Fig. 31). To confirm that the observed GFP-associated vesicular structures were indeed lipid droplets, transgenic animals were stained with the lipophilic reagent LipidTox as previously described (O'Rourke et al., 2009). The translational GFP fusion protein reporters were localised at the periphery of fat droplets that were LipidTox positive.

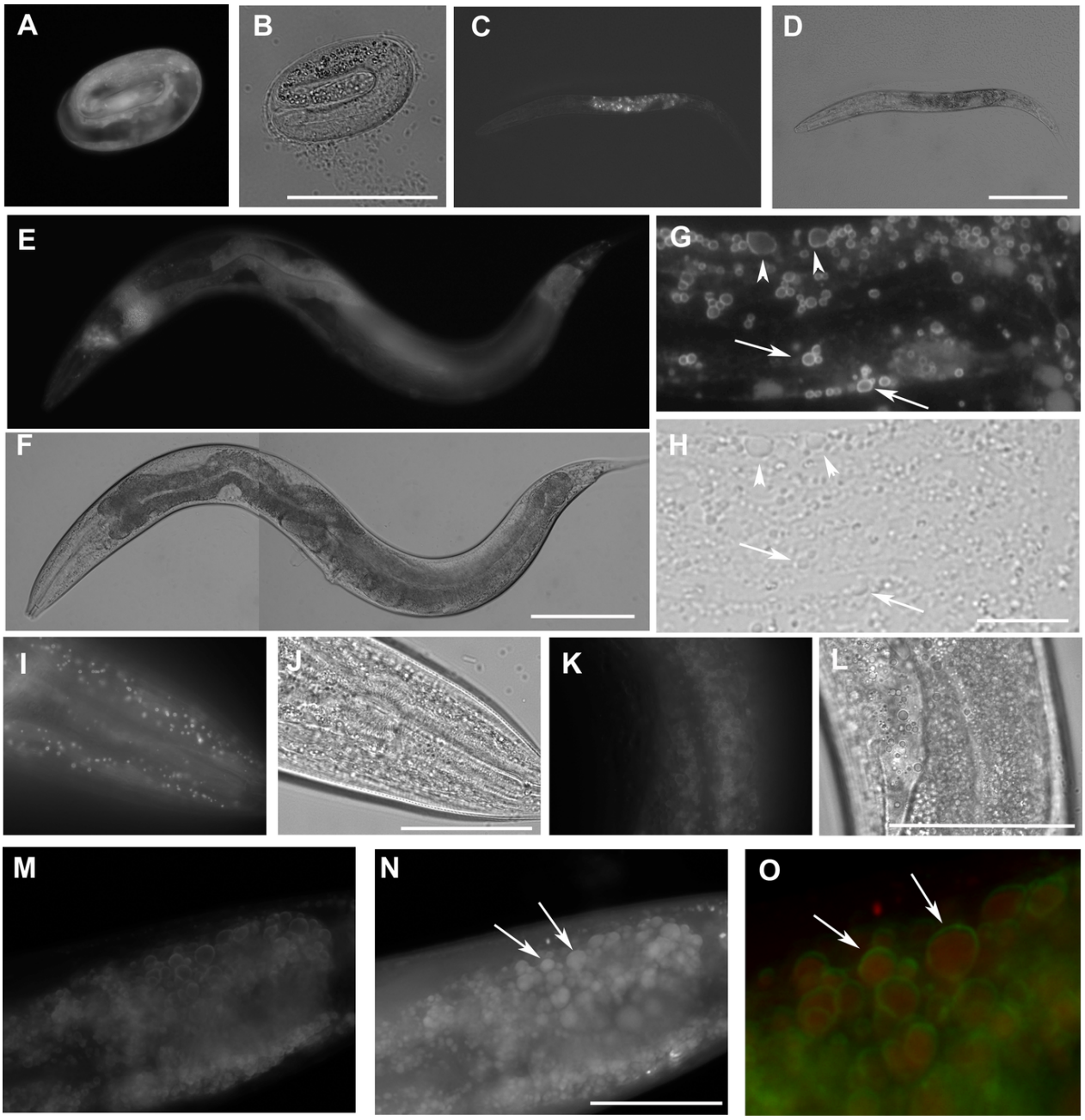


Figure 31

The expression W01A8.1::gfp reporter genes in transgenic strains. W01A8.1a/c::GFP is shown in (A, C, E, G), and (I), and corresponding areas in Nomarski optics are shown in (B, D, F, H) and (J). (A) The onset of expression of W01A8.1a/c::GFP in epidermal cells and in intestinal cells of the three-fold embryo. (C) The expression of W01A8.1a/c::GFP in intestinal cells of an L2 larva. (E) and (G) W01A8.1a/c::GFP expression in epidermal cells and intestinal cells of a young adult hermaphrodite. (G) GFP fluorescence around lipid droplet-like structures in the intestine that are marked by arrows and arrowheads. The corresponding image in Nomarski optics is in (H). (I) A higher magnification the lipid droplet-like structures in epidermal cells labelled by W01A8.1a/c::GFP (shown in Nomarski optics in the J). (K) Lipid droplets of an unfixed intestine labelled by W01A8.1b::GFP (corresponding Nomarski image is in L). (M, N) and (O) Part of the intestine of an adult larva expressing W01A8.1b::GFP (M) with the corresponding staining of lipid droplets by LipidTox (N). (O) LipidTox-positive lipid droplets (red) with W01A8.1b::GFP on the periphery (green) in this merged view. Bars represent 50 μm in (B, H, J, L) and (N) and 100 μm in (D) and (F).

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 localised on spherical cytoplasmic structures primarily in gut and epidermal cells (Figs 32. A, C, D and F) 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 (Figs 32. G and I). The structures clearly labelled with PLIN1::GFP and PLIN2::GFP were also positive in LipidTox staining (shown for PLIN2::GFP in Figs. J – L). We conclude that W01A8.1 is localised on the same structures as human PLIN1 and PLIN2.

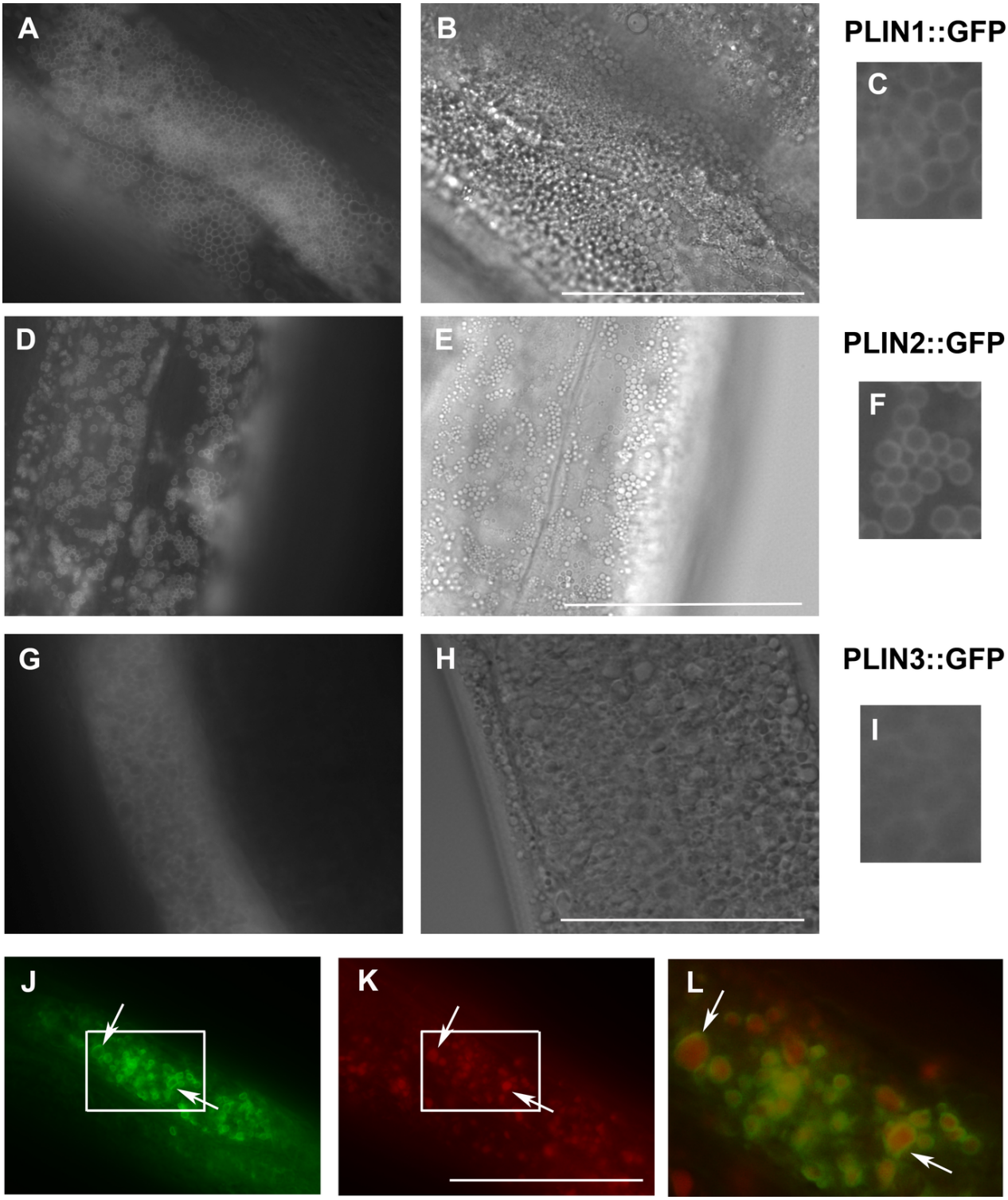


Figure 32

Expression of human perilipins fused to GFP in C. elegans. (A–C) Expression of human PLIN1::GFP in live transgenic *C. elegans*. PLIN1::GFP is localised on vesicles with an appearance of lipid droplets. PLIN2::GFP (D–F) is localised in transgenic animals on vesicular structures with an appearance of lipid droplets similarly as PLIN1::GFP. PLIN3::GFP (G, H and I) yields a more diffuse cytoplasmic pattern with faintly stained vesicular structures. (A, D) and (G) and details in (C, F) and (I) show GFP in fluorescence microscopy and (B, E) and (H) corresponding areas to (A, D) and (G) in Nomarski optics. (J, K) and (L) show PLIN2::GFP in fluorescence microscopy (J) in fixed C. elegans stained with LipidTox (K). The area indicated by the white rectangle in (J)

and (K) is magnified and merged for co-localization of PLIN2::GFP (green) and LipidTox (red) in (L). Arrows indicate lipid droplets clearly marked by GFP with the LipidTox positive content. Bars represent 50 µm.

W01A8.1 knockdown alters the appearance of lipid droplets in early embryos and has an effect on brood size

To test the function of W01A8.1, 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 (n = 260, n = 550, for day one and n = 1,000, n = 1,400 for day two).

Repetition of knockdown by RNAi feeding over two generations confirmed this observation. dsRNA feeding caused the W01A8.1 specific group to produce ~30% fewer larvae compared to controls, the experiment was repeated twice independently with consistent findings. We confirmed, using RT-qPCR, that feeding based knockdown resulted in approximately 45% decrease in *W01A8.1* transcripts (data not shown).

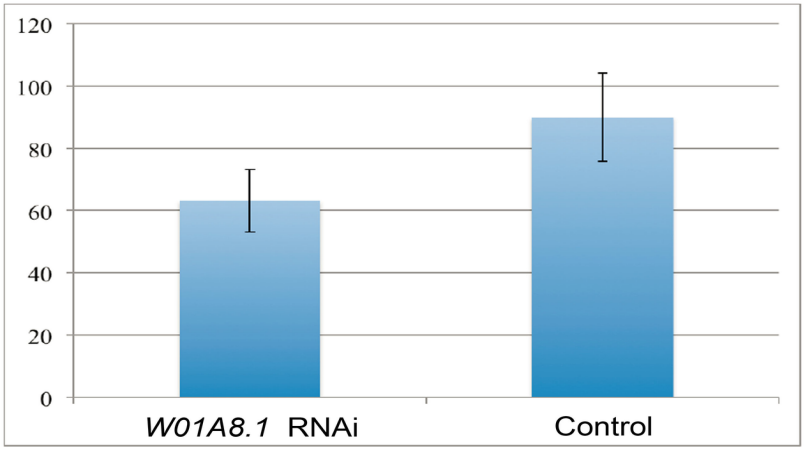


Figure 33

Analysis of the number of progeny after W01A8.1 gene inhibition by RNAi. The graph shows the knockdown effect of W01A8.1 gene on the amount of progeny compared to controls. Wild type animals were treated with RNAi (dsRNA feeding) directed against W01A8.1 for two generations and the amount of progeny was determined during a 24-hour period per one parent animal (21 parent animals were scored in both categories). The data presented here shows (y-axis) the average number of progeny per parent, n = 1,278 and n = 1,813, respectively, $p < 0.05$. The SD is indicated.

Staining of adult hermaphrodites with LipidTox (after formaldehyde fixation) revealed larger lipid droplets in early embryos derived from adults inhibited for W01A8.1 (Figs 34. A and B) compared to controls (Figs 34. C and D).

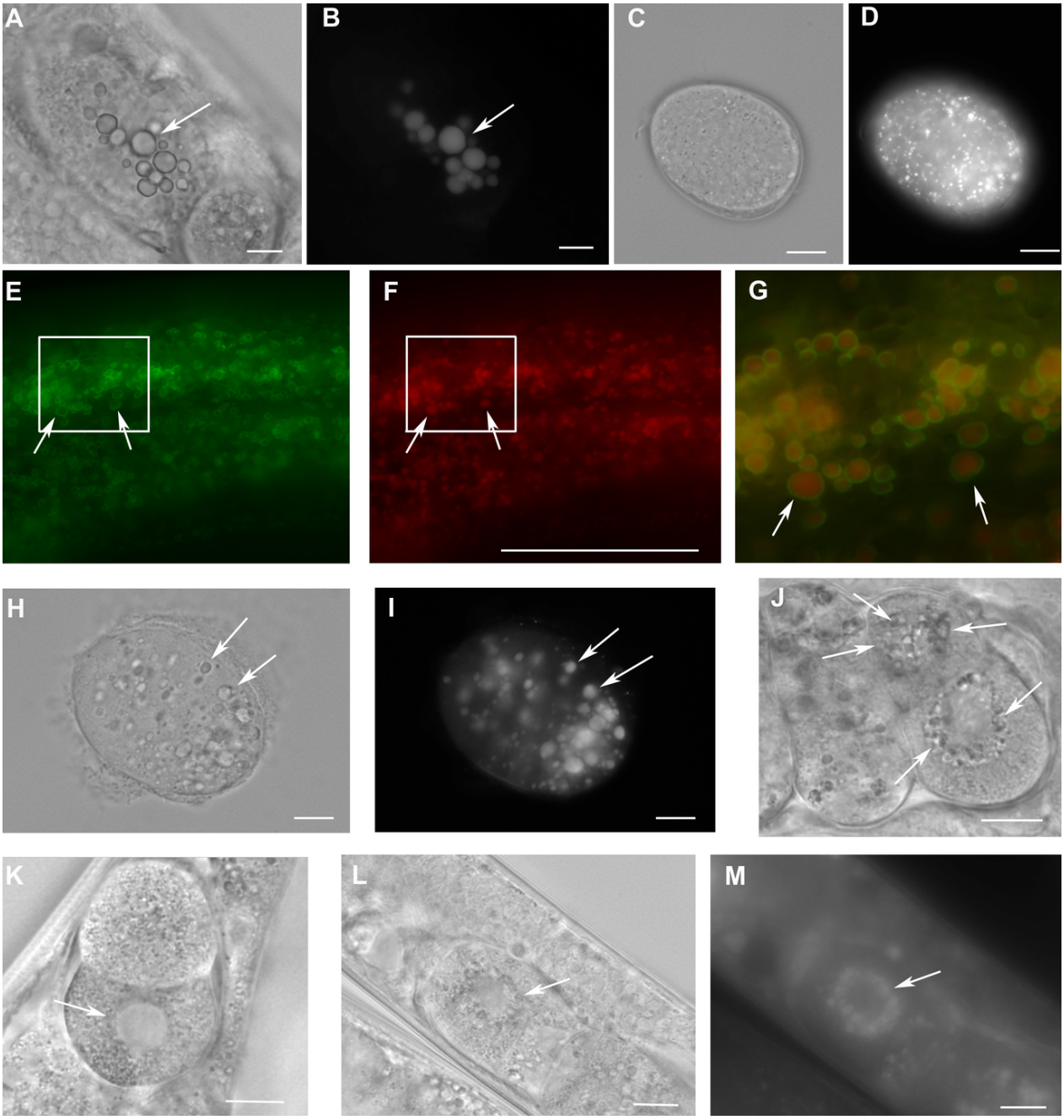


Figure 34

Loss of W01A8.1 function results in abnormal lipid droplet appearance. (A) and (B) An embryo from a hermaphrodite inhibited for W01A8.1 function by RNAi. Large lipid droplets stained by LipidTox (B) are visible also in Nomarski optics (A) in contrast with a control embryo that has only small and more evenly distributed lipid droplets (C–Nomarski optics and D–LipidTox staining). (E–J, L) and (M) Images of structures observed in animals with disrupted W01A8.1. (E) and (F) show structures with the appearance of lipid droplets in the intestine of an animal with disrupted W01A8.1 balanced with the synthetic transgene W01A8.1(synth)::gfp. GFP tagged synthetic W01A8.1a is localised on lipid droplets-like vesicular structures (E). (F) Shows the same area stained with LipidTox. (G) Shows in magnification a merged image of the area indicated by white rectangles in (E) and (F). Arrows indicate W01A8.1(synth)::GFP labelled lipid droplets (green) positive for lipids in LipidTox staining (red). (H) and (I) show an embryo of a parent with disrupted W01A8.1 that had confirmed a loss of the balancing transgene. Large LipidTox stained droplets are visible in Nomarski optics (H) as well as in LipidTox staining (I). Their enlargement is clearly visible in

comparison with the wild type embryo shown in (C) and (D). (J) and (K) are images of live animals. (J) Shows an embryo with disrupted W01A8.1 and confirmed loss of the balancing transgene. Large vesicular structures are formed around the dividing nucleus (arrows). (K) Shows a control embryo with normal appearance of the nuclear periphery (arrow). (L) and (M) show a one cell embryo from a parent with disrupted W01A8.1 and confirmed loss of extrachromosomal array containing W01A8.1(a)synth::gfp after fixation and staining by LipidTox with large lipid droplets around the dividing nucleus visible in Nomarski optics (L) and positive for lipids in LipidTox staining (M) indicated by arrows. Bars represent 10 μm.

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

In order to eliminate the W01A8.1 function completely, we designed a CRISPR/Cas9-mediated gene editing approach to eliminate almost the entire coding region.

We also included a rescuing plasmid consisting of isoform a that was prepared as cDNA synthesized in vitro using synonymous codons (W01A8.1(a)synth::gfp) that is protected against CRISPR/Cas9 targeted editing but allows the production of the wild type isoform a at the protein level.

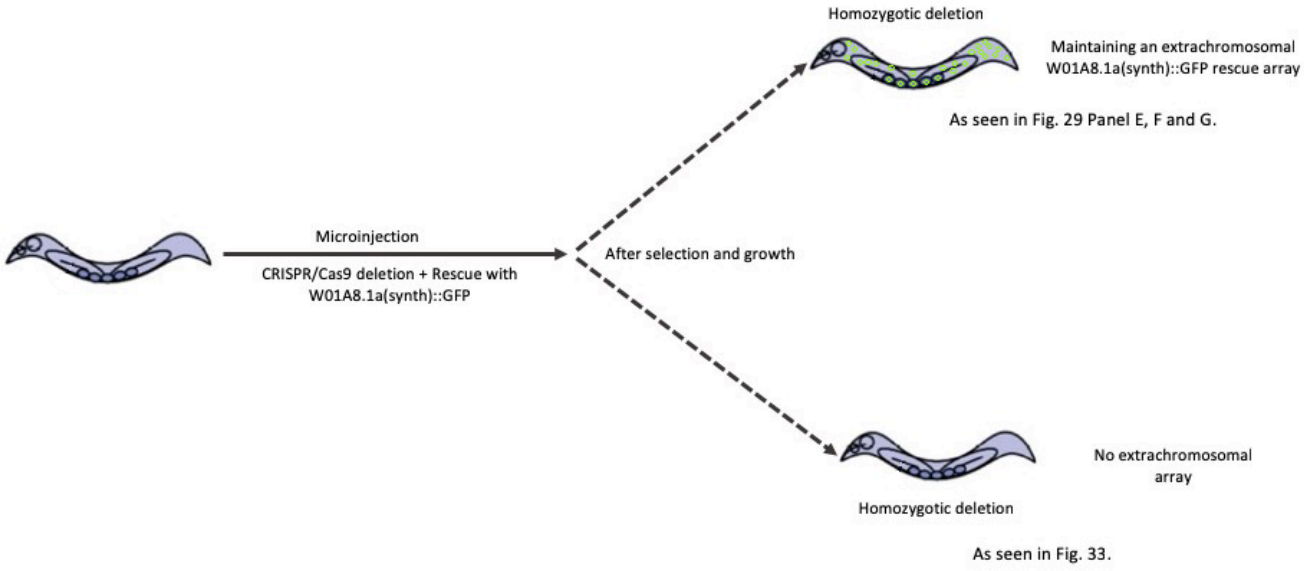


Figure 35

Outline of the procedure carried out to obtain animals with disrupted W01A8.1 gene and worms with a rescue array. A PCR confirmation of the deletion is shown below. A PCR confirmation of the deletion is shown below.

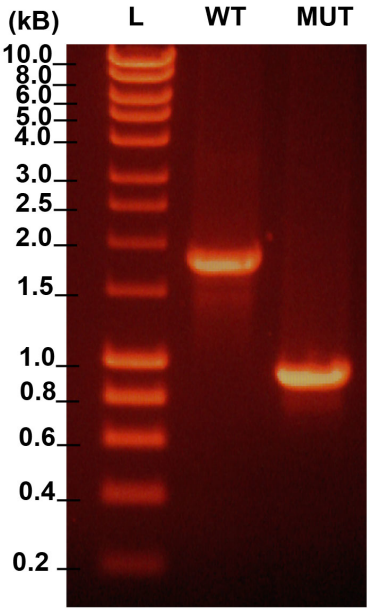


Figure 36

Analysis of W01A8.1 gene by PCR in wild type animals and animals subjected to CRISPR/Cas9 targeted gene deletion. Specific primers efficiently amplify full length fragment in wild type animals (WT) while mutant animals show homozygous deletion in W01A8.1 gene.

Lines that expressed the GFP fusion transgene were morphologically normal and W01A8.1(a)synth::GFP was found on lipid droplet-like structures as expected (Figs 34.E and F) that also stained positive by LipidTox (Figs 34.F and G). This transgenic strain yielded lines either carrying or losing the rescuing transgene in the background of a disrupted endogenous W01A8.1. The elimination of W01A8.1 was easily monitored by PCR (Fig. 36). Surprisingly, animals with the deleted W01A8.1 locus that lost the extra-chromosomal rescuing array were able to reproduce normally. From several lines that had a confirmed disruption of W01A8.1 and a confirmed loss of the extrachromosomal array, the line CK123 (KV001) was selected and used for subsequent analyses. As was observed in W01A8.1 RNAi embryos, loss of W01A8.1 activity resulted in the formation of large LipidTox-positive structures (Figs 34.H and I) that were clearly bigger than droplets observed in control embryos using the same protocol (Figs 34. C and D). These large lipid-containing structures were observable in live mutant embryos (Fig 34.J) but not in wild type embryos (Fig 34.K) using Nomarski optics. Viewing through multiple focal planes in live, 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 dividing nucleus (Figs.34L and M). 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.

To visualise lipid-containing structures in *W01A8.1* null mutants and wild-type *in vivo*, we used CARS microscopy (done with kind help of Dr. Zhongxiang Jiang, Leica Microsystems, Mannheim, Germany). 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 (Figs. 37A and B), the sudden re-localisation of these structures to the periphery of the dividing nucleus in the first embryonic division (Fig. 37E), and the propagation of this phenotype, although with gradually diminishing appearance, throughout embryonic development (Figs. 37F and H). 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 (Figs. 37C, D and G) than in W01A8.1 null embryos. In contrast to embryos, lipid-containing structures in intestinal and epidermal cells of adult W01A8.1 null mutants (Figs. 37J and L) were smaller than lipid-containing structures in control animals (Figs. 37I and K).

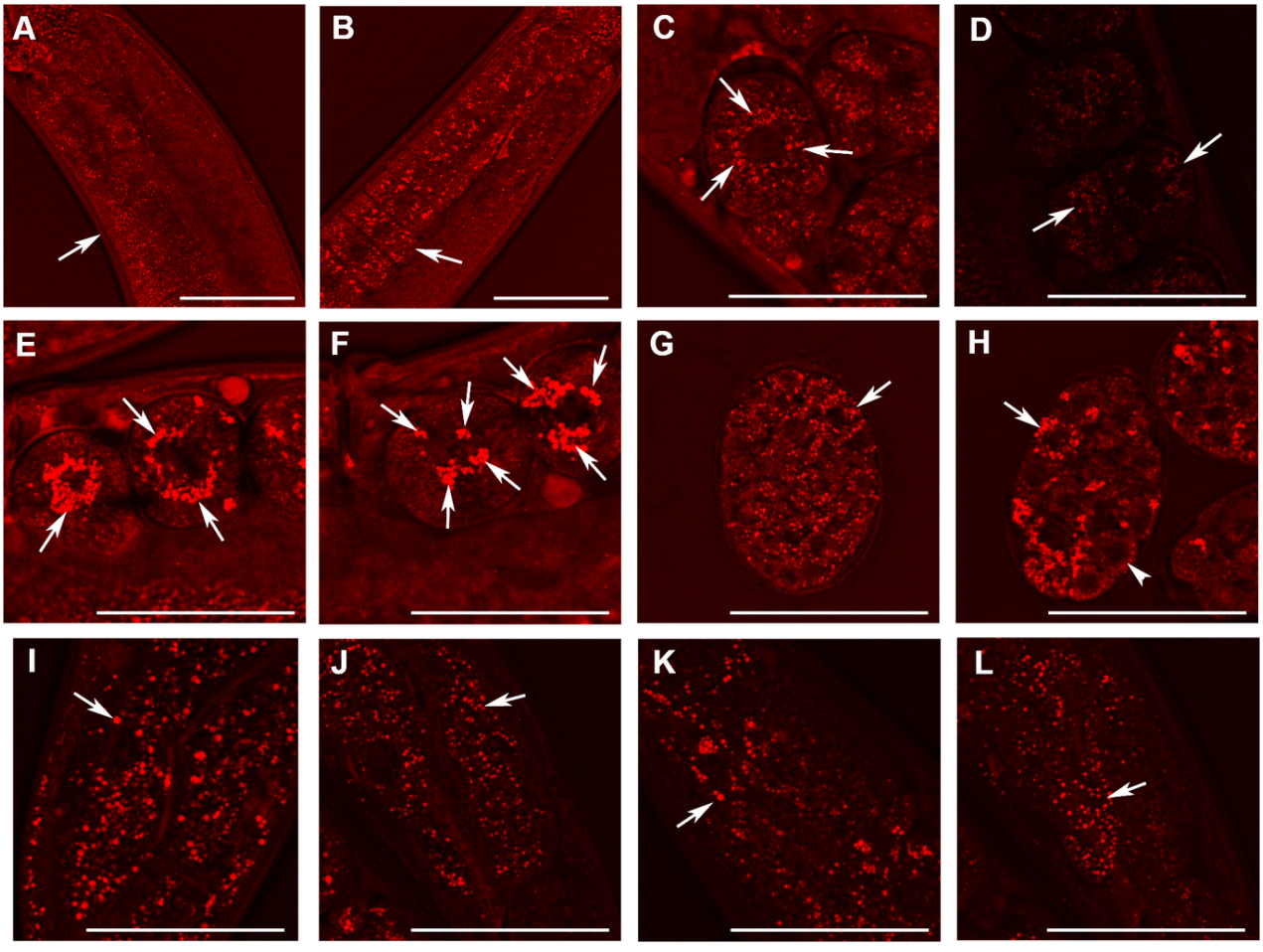


Figure 37

Analysis of lipid containing structures in live wild type animals and in animals with disrupted W01A8.1 by CARS microscopy. CARS microscopy was performed using constant magnification and intensity settings (20% laser intensity) with the exception of control embryos (C and G) that were examined at 30% laser intensity since the lipid content was lower in wild type. The brightness of the entire figure was digitally

enhanced using Adobe Photoshop brightness setting (+150 units) for better visibility of structures. Arrows indicate lipid containing structures in paired panels. (A) The germline of a wild type adult hermaphrodite animal with small lipid containing structures in oocytes and an increase in their number and size during oogenesis. (B) The germline of a mutant hermaphrodite animal. Lipid containing structures are bigger compared to the control animal yet distributed evenly in mutant oocytes. (C) Lipid containing structures localize around the nucleus in one cell wild type embryos. This tendency of the association of the lipid containing structures with nuclear periphery can be seen also during later developmental stages in wild type embryos (D). (E, F) and (H) Enlarged lipid containing structures arranged around the nuclei in mutant embryos. (F) The formation of clusters of lipid containing structures on the periphery of nuclei. (G) Shows a wild type embryo at later stage of the development. (H) The lipid containing structures progressively diminish in size in mutant embryos during later stages of embryonic development (arrowhead). (I) and (J) Lipid containing structures in enterocytes of wild type (I) and mutant (J) animals. In contrast to embryos, which exhibit higher CARS signal and bigger lipid containing structures in mutant animals, gut cells in adult animals show the opposite, that is, a reduced fat-related CARS signal and smaller lipid containing structures in mutant animals. Similarly, lipid containing structures in epidermal cells (K and L) are bigger in wild type animals as shown in the (K) and smaller in mutant animals (L). Bars represent 50 μm.

The morphometric analysis confirmed that null mutant embryos of *W01A8.1* contained larger lipid containing structures as observed by CARS microscopy (Figs. 37A and B) that represent a larger total area (Fig. 38C), as determined by quantifying individual focal planes images. Moreover, morphometry revealed many smaller structures with area 1–4 AU (arbitrary units) with given threshold settings (Figs. 38A and E). There was an obvious inverse correlation in the number of larger and smaller structures (with area <5 AU) between embryos and somatic tissues. The analysis in Figs. 38B and F shows that inclusion of small structures into analysis does not significantly affect the results but affects only the 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.

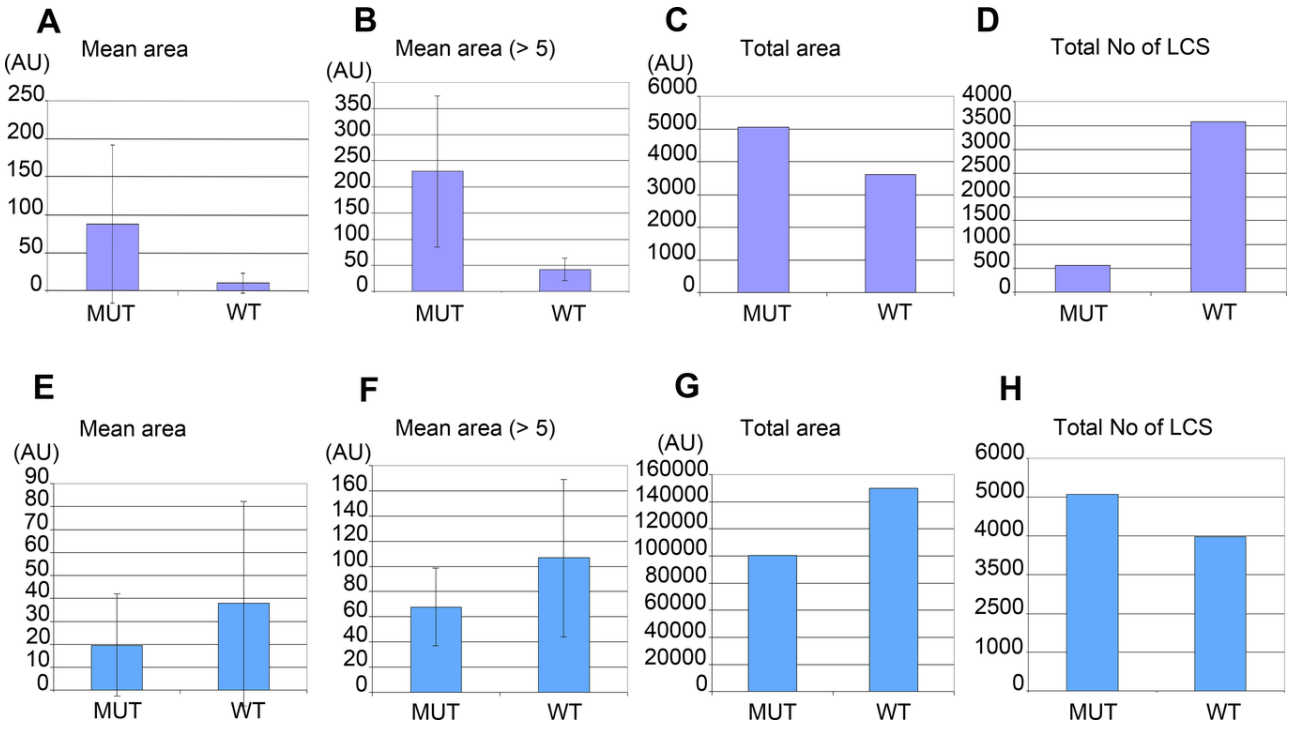


Figure 38

Morphometric analysis of lipid-containing structures by CARS microscopy in wild type and W01A8.1 null animals. Morphometric analysis was performed on CARS positive structures in single focal plane images acquired from representative mutant and wild type animal image stacks. (A-D) compare early embryos from mutant (MUT) and wild type (WT) animals using seven representative CARS images. (A) shows the mean area of all individually recognizable structures and (B) shows the mean area of all individually recognizable structures with an area bigger or equal to 5 arbitrary units (derived from pixels at the same settings). (C) compares the total area of all CARS positive structures in mutant and wild type embryos while (D) compares the total number of individually recognizable CARS positive structures (lipid-containing structures–LCS) in the same embryos. (E-H) compare adult somatic tissue (tail region) from mutants and wild type hermaphrodites using five representative CARS images. (E) shows the mean area of all individually recognizable CARS positive structures and (F) shows the mean area of all individually recognizable structures with an area bigger or equal to 5 arbitrary units. (G) compares the total area of all CARS positive structures in mutant and wild type tail regions while (H) compares the total number of individually recognizable CARS containing structures (LCS) in the same regions. Vertical bars in (A, B, E) and (F) represent Standard Deviation. The results presented in (A, B, E) and (F) are statistically significant in two-tailed Student's t-test ($p < 0.0001$).

Despite the fact that there were a larger number of individually recognizable lipid-containing structures in wild type embryos (38D), the mean area of these structures in mutants was considerably larger (38C). In contrast, adult mutant animals contained smaller, more numerous lipid-containing structures (38E, F and H) that covered a smaller total area (38G) (and therefore volume) compared to wild type controls. The morphometric analysis confirms that there is more CARS positive signal and therefore most likely more fat in *W01A8.1* null embryos (despite lower threshold used for analysis of wild type embryos) and reduced CARS positive signal (and less fat) in adult tissues of *W01A8.1* null animals compared to controls.

The analysis of the number of progeny laid by animals lacking W01A8.1 in comparison to wild type animals showed a decrease of progeny in mutant animals statistically significant on day 3 (Fig. 39).

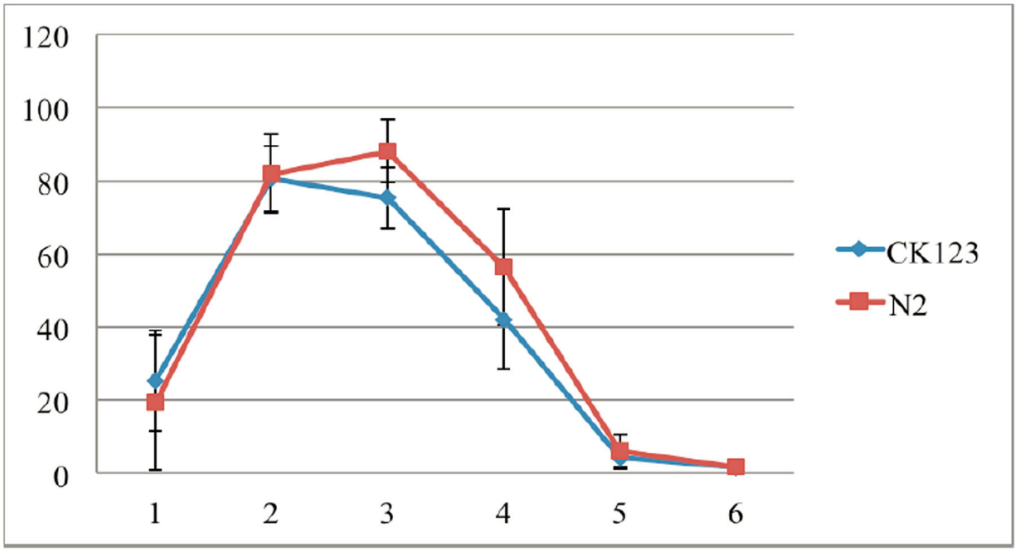


Figure 39

Analysis of the number of progeny laid by mutants with disrupted W01A8.1 and confirmed loss of the extrachromosomal array (CK123, KV001) and control N2 animal. The values show a mild statistically significant difference in the number of progeny laid by mutant worms CK123 compared to wild type N2 animals during day 3 (Two-Tailed T-Test, p = 0.04962). Bars indicate SD.

Results of Part III

Here I present the summary of the results from the third publication, which is relevant to the discussion of this dissertation. This section was modified under the Creative Commons CC-BY 4.0 license from Kostrouchová et al., (2017). The details of the results can be found in the original article.

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 (e.g., human MED28_HUMAN and *D. melanogaster* MED28_DROME). 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. BLAST and PSI-BLAST searches in their standard settings were not able to reveal a significant hit ($E < 10^{-3}$); the only nematode hit was a *Trichinella spiralis* protein (E5RZQ1). However, when the 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.

All PSI-BLAST MED28 homologues possess variable N- and C-termini of 3–80 amino acids showing no conservation across Metazoa. This conservation is loose even just within *Drosophilae* or *Caenorhabditae* sequences. Only the central core of about 110 amino acids is preserved in metazoan evolution. Figure 36 shows a sequence alignment of this conserved core of selected MED28 homologues. All sequences are predicted to fold into three helices forming a putative coiled-coil fold (UniProt annotation). Submitting the alignment to HHpred for 3D structure recognition reveals a structural fold of yeast MED21 (PDB identifier 1ykh_B). It is indeed a three-helix coiled-coil forming a heterodimer with MED7. It can be expected that MED28 forms a very similar fold interacting with a yet to be determined subunit of the MED complex.

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

According to the genetic data from WormBase, it is suggested that the *F28F8.5* gene can be expressed as both an individual and multi-gene transcript, located as the last gene in a four-gene operon (CEOP5444) that is both SL-1 and SL-2 trans-spliced.

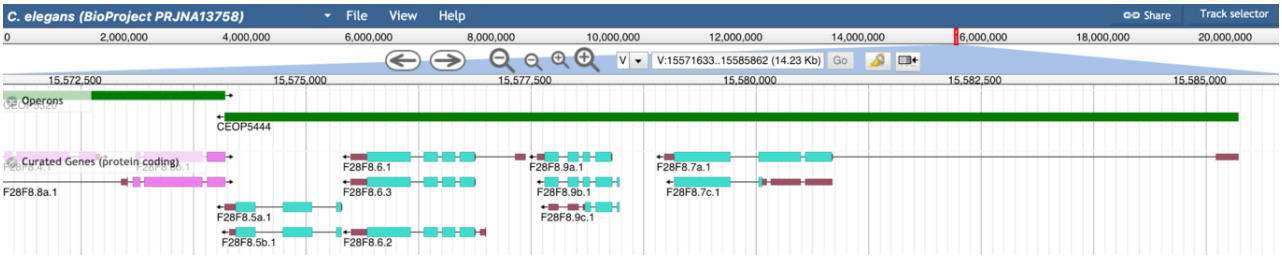


Figure 40

Image taken from WormBase Genome Browser tool. The operon CEOP5444, marked with a green solid line, showing the four genes under its regulation. F28F8.5 can be seen as the last gene in the operon.

To determine the intracellular localisation of F28F8.5, we edited the *F28F8.5* gene using CRISPR/Cas9 technology. We inserted the gene coding for GFP directly in front of the first codon. The arrangement used in our experiment employed a self-excising cassette (SEC) that was added after *gfp*. This genetic editing strategy is based on several publications (Dickinson et al., 2013; Ward, 2015; Dickinson et al., 2015; Dickinson and Goldstein, 2016), for technical details I refer to the original article.

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, based on the assumption that the tag modification the genome doesn't affect the expression of the other genes in the operon.

By heat shock we induced 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 Rol 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. Note that both known protein isoforms of F28F8.5 (a and b) would be tagged on their N-terminus with GFP by this method.

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 microscopy was used to determine the subcellular distribution of GFP::F28F8.5. Confocal scanning through multiple focal planes 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. The principle is based on the fact that that 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.

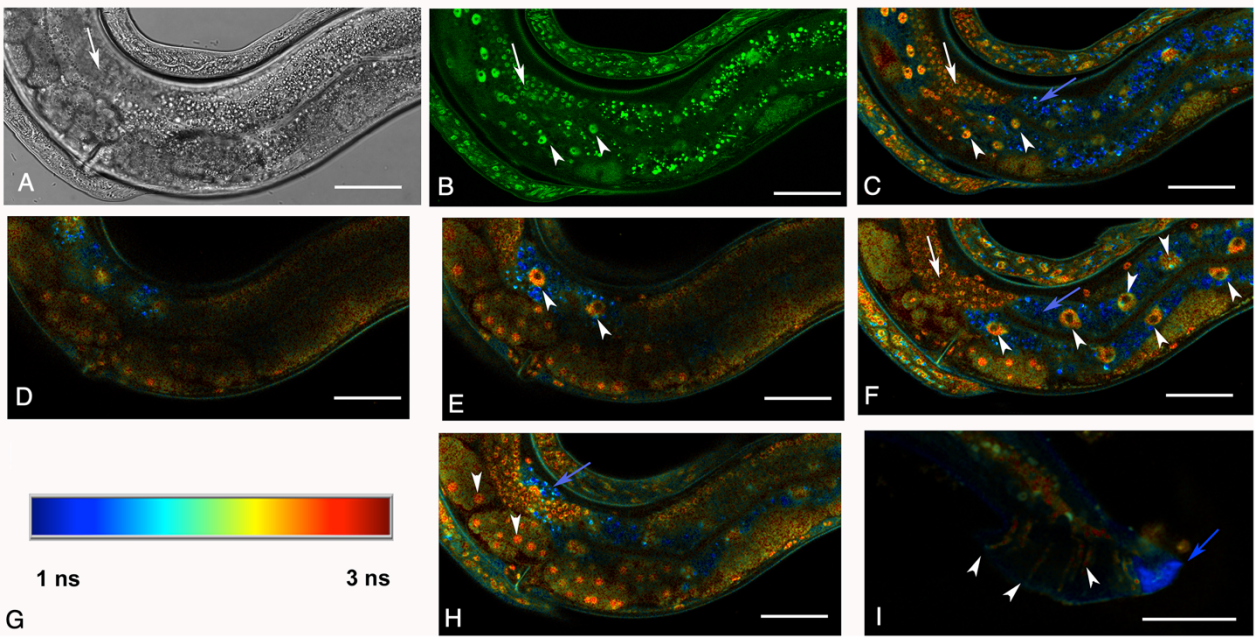


Figure 41

Analysis of GFP::F28F8.5 expression in homozygous animals with edited F28F8.5 gene by confocal microscopy and fluorescence lifetime imaging microscopy (FLIM). All confocal images of GFP fluorescence are recorded in Channel 1 (495-525 nm). FLIM images (panel C, D, E, F, H, and I) are calculated from merged recordings in Channel 1 and Channel 2 (525-585 nm). Panels A image was recorded in Nomarski optics at the same optical focus as in corresponding confocal images of GFP fluorescence (panel B). Arrowheads indicate nuclei of enterocytes. Panels D to 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. 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.

F28F8.5 interacts with Mediator complex subunits and regulates development

To truly understand the relationship between our newly identified gene *F28F8.5* and the Mediator complex's other members we attempted to determine the in vitro interaction of the protein F28F8.5 with known members of the complex. 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, due to the technical difficulties we were unable to produce the labelled protein in the rabbit reticulocyte system. Therefore, 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 (Baudino et al., 1998; Barry et al., 2003; Fantappié et al., 2008) 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; Leong et al., 2004). 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; Libý et al., 2006).

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). 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*. As described in the sections above (*Trichoplax adhaerens* as a model organism), genetic analysis has revealed that *T. adhaerens* is one of the most basal diploblastic species of metazoans known with a highly conserved set of NRs. This very concept as well *T. adhaerens'* evolutionary position 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 support 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 early metazoan evolution and developmental regulatory needs of increasingly more complex metazoan species. Keeping with our published finding were there data provided by Reitzel et al., (2018). They concluded supporting the hypotheses that the original RXR protein in metazoans bound a ligand with structural similarity to 9-*cis*-retinoic acid; the DNA motif recognised by RXR has changed little in more than 1 billion years of evolution and the suite of processes regulated by this transcription factor diversified early in animal evolution.

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

As it had already been 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 very likely 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; Conaway and Conaway, 2011a; Conaway et al., 2005; 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 Kuang-Lei Tsai (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* 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. The figure below, taken from the publication, highlights the two domains.

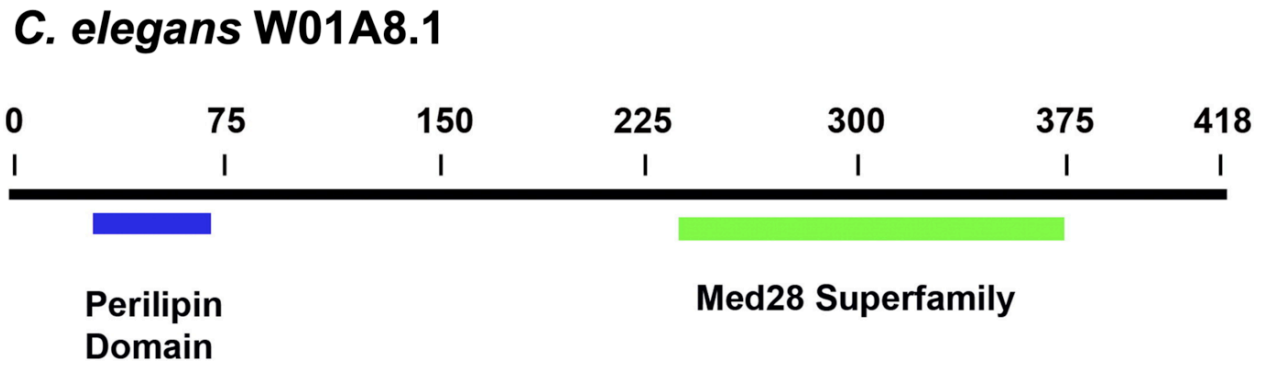


Figure 42

Image adopted under Creative Commons license from Vrablik et al., (2015) showing Conserved superfamily domains of *C. elegans* W01A8.1.

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. Below is the figure (adapted with permission) from the publication showing that the LD targeting domain partly overlaps the so-called 'MED28 superfamily' domain. 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.

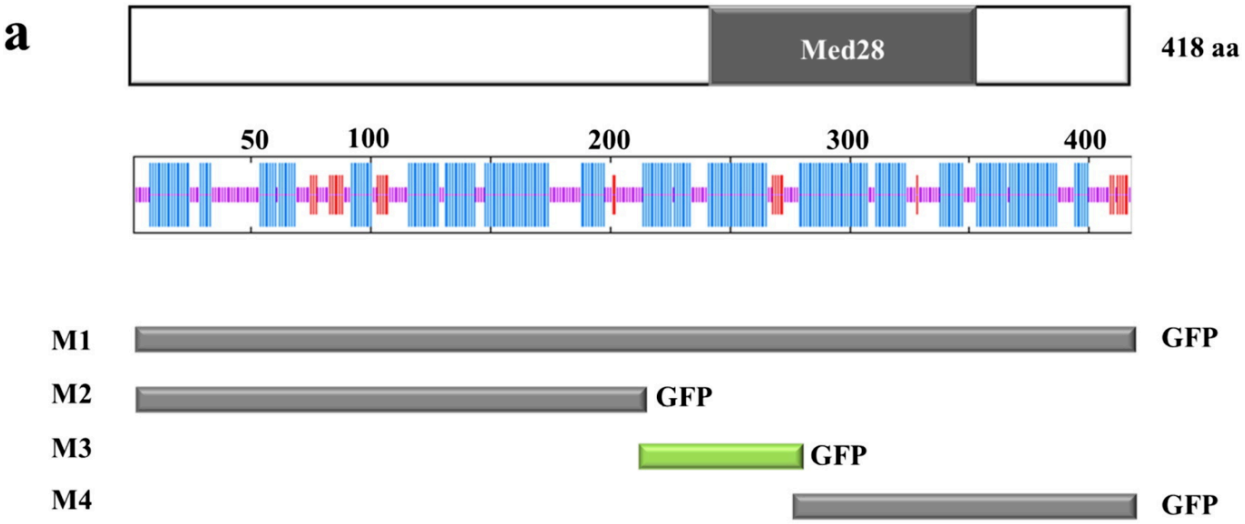


Figure 43

Figure adopted with permission from Na et al., (2015) showing various showing the protein sequence with relation to hydrophobicity. Truncations of W01A8.1 were made based on hydrophobicity (indicated with red vertical lines) and potential alpha helices (indicated with blue vertical lines). M1-M4 are the different truncated proteins that were fused with GFP, expressed in Chinese hamster ovary (CHO K2) cells, and co-imaged with LipidTox staining using confocal microscopy. The M3 peptide sequence containing the amino acids 211 through 275 was able to target GFP around LDs.

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 at Perilipin from an evolutionary point of view, 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. The 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 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. A commonly used dye Nile red is a phenoxazone, lipophilic dye, derived from Nile blue that is concentrated in hydrophobic environments when used *in vivo* or in cell lines (Fowler and Greenspan, 1985; Greenspan et al., 1985). In hydrophobic environments, Nile red undergoes an increase in yellow-gold fluorescence, making it a useful marker of intracellular lipid droplets (Bonilla and Prelle, 1987; Fowler et al., 1987). Other examples of dyes used include BODIPY-labeled fatty acids which have similar properties when used in cell cultures. Both stains have been used as vital dyes on living cell cultures and tissues, or, in the case of *C. elegans*, on living animals. Nile red and BODIPY-labeled fatty acids stain living worms when the dyes are mixed with their food, *E. coli* bacteria (Ashrafi et al., 2003). Many researchers involved in the field of *C. elegans* metabolism have extensively used these fluorescent dyes to assess fat stores, as they stain vesicular structures in *C. elegans'* main fat storage organ, the intestine. Several publications have used Nile red as a proxy for fat content in *C. elegans*, accrediting a lipid regulatory role to many genes with an altered Nile red staining phenotype. In many studies, Nile red poorly stains the germline, eggs, and hypodermis, tissues which are known to be high in lipid content by other methods. Furthermore, for certain mutant worms (*daf2* mutants), lipid storage revealed by biochemical analysis showed paradoxical findings to those obtained with Nile red or BODIPY-fatty acids staining (Soukas et al., 2009; Wang et al., 2008). 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 to us 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 often difficult to achieve complete penetrance of the fixative and/or stain in all worms and tissue. This was very often the case for embryos, which are covered with the infamous chitin layer, making the staining process notoriously unreliable. And in spite of proper fixation and staining, several fixation artefacts were 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.*

If W01A8.1 was truly a Perilipin family member, then the compartment it encapsulates should theoretically be also labelled by Perilipins form other species in *C. elegans*. Another group ran similar experiments and expressed *Drosophila* PLIN1::GFP in *C. elegans* to address whether it can mark lipid droplets, act as a fat storage indicator, reveal tissue-specific fat storage patterns and serve as a marker to identify fat storage regulatory genes (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 (Fig. 32) each marked with GFP using a natural promoter of W01A8.1. We used an extrachromosomal plasmid-based approach and injected the plasmids into the *C. elegans* gonads and then selected for positive progeny. We were actually relatively successful in selecting the worms carrying the arrays. This may perhaps be an indication that the slight overexpression was not toxic to cells. Nevertheless, 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, it 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 i.e. not associated to LDs, 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. 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), shown in Fig. 43, indicates that the amino acids 211 through 275 of W01A8.1 are responsible for the targeting to LDs. This region, based on our alignment (Fig. 25), 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 W01A8.1 or rather a Perilipin-related protein 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 MED28 superfamily domain.

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

To see the effects of W01A8.1 knock-down as well as knock-out on *C. elegans* RNAi and CRISPR/Cas9 mediated deletion approaches were employed. 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 (Bell et al., 2008; Bulankina et al., 2009). Data from *Drosophila* suggests that Perilipin deletion mutants are viable but proper lipid homeostasis in flies is lacking (Beller et al., 2010; Bi et al., 2012).

We conducted RNAi experiments using the injection and feeding method, 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 not only a 45% knockdown could be quantified using RT-qPCR for the feeding experiments. We observed a reduced brood size of 30% fewer progeny and clumped lipid droplets in the germ line. Furthermore, we proceeded to create a null mutant using the aforementioned CRISPR/Cas9 technique. The morphological phenotype of the mutant was consistent with what was observed by RNAi, however, we didn't observe any significant differences 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 our) from Na et al., (2015) and Vrablik et al., (2015).

We had already realised the experimental limitation of lipid assessment in *C. elegans* when using fixative dyes so we turned to Coherent anti-Stokes Raman Scattering (CARS) microscopy for a much more precise phenotypical analysis. CARS microscopy literally and figuratively shed new light on our understanding of W01A8.1. When it is thought of as a true orthologue of Perilipin then one can begin to explain the phenotypes seen. Analysis of lipid-containing structures in developing embryos and in adult tissues suggested that W01A8.1 protein isoforms could differ in embryos than in adult tissues and/or lipid-containing structures in embryos are likely to differ from those of adult tissues, additionally other cell-specific mechanisms could be responsible. 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 reported in hepatitis C virus (HCV) infected cells (Boulant et al., 2008). Essentially 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 regards 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.

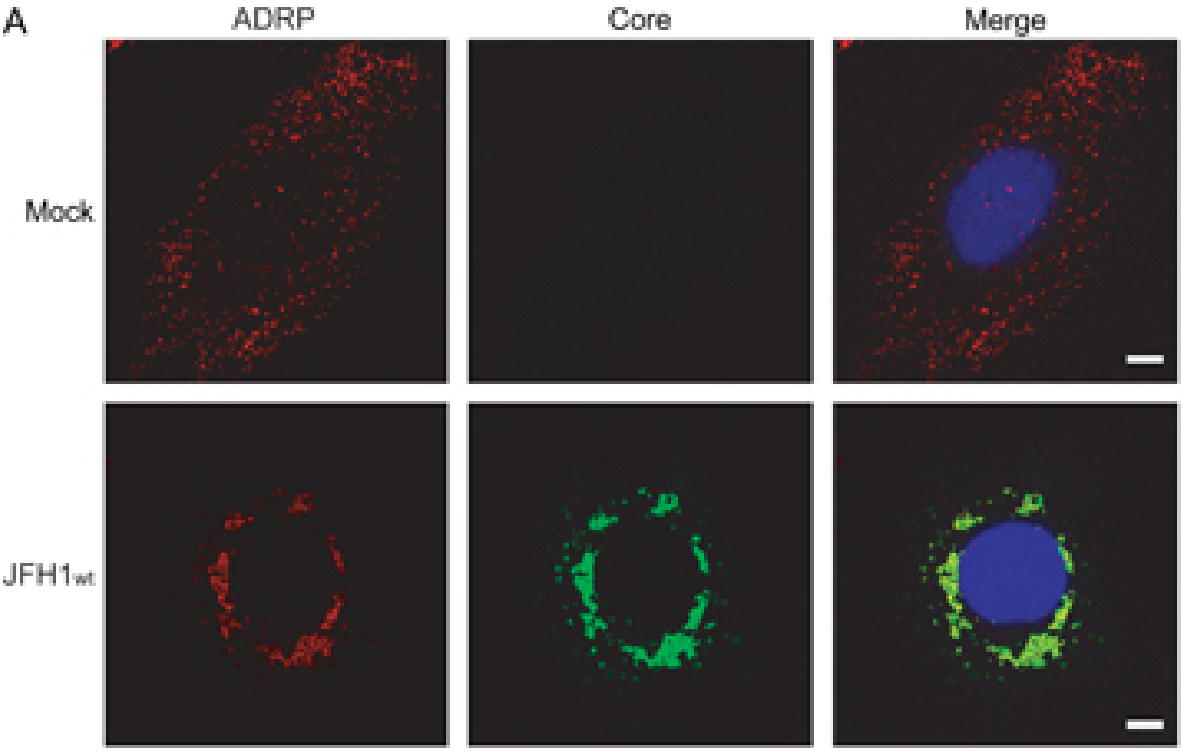


Figure 44

Image taken with permission from Boulant et al., (2008) shown hepatitis C virus Core Protein induces lipid droplet redistribution in a microtubule- and dynein-dependent manner.

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). They showed that Perilipin is specifically required for the regulated transport of lipid droplet in *Drosophila*. 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 found 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

At this point, one can be convinced that W01A8.1 can be a very promising candidate for a Perilipin homologue in *C. elegans*. However, to be Perilipin it also has to have an effect on lipid content and not just distribution. To put things in context, below is an illustration showing the basic lipolytic process involving key players.

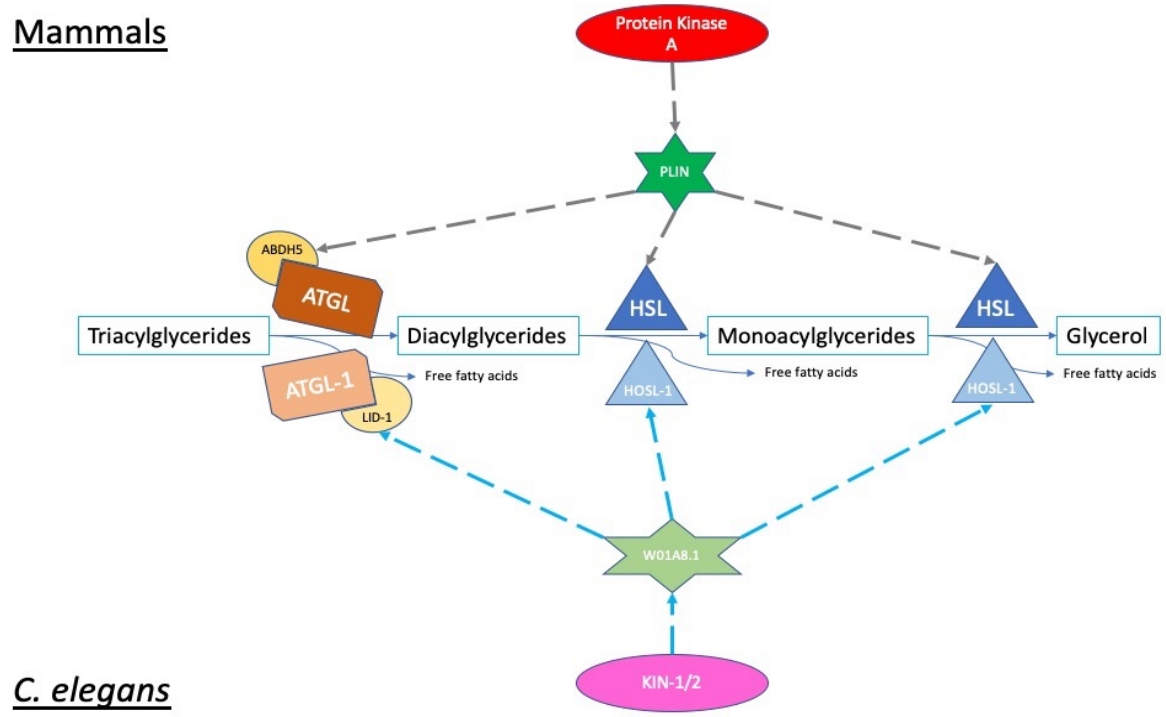


Figure 45

Enzymes and regulatory proteins involved in lipolysis (Adapted from Lass et al., (2011)). Mammalian proteins are indicated above and their C. elegans orthologues below. Triacylglycerides (TAG) are progressively hydrolysed to diacylglycerides (DAG), monoacylglycerides (MAG) and glycerol (G) by lipases for each of

these steps: adipose triacylglycerol lipase (ATGL) and hormone-sensitive lipase (HSL). LID-1 is proposed to be orthologues of ABHD5/CGI58 in *C. elegans*. The access of ATGL and HSL to lipid droplets is regulated by Perilipin, which is under the control of protein kinase A (PKA) Xie and Roy, (2015); Lee et al., (2014). W01A8.1 is proposed to be a Perilipin orthologue in the present work.

CARS microscopy was also 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 larvae (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 speaking, 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 found in absence of W01A8.1 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 Perilipin than to MED28. Furthermore, Perilipins were also implicated in the regulation of lipophagy (Kaushik and Cuervo, 2015; Kaushik and Cuervo, 2016). With regards to lipophagy as an alternative pathway in *C. elegans*, we have also discussed and proposed this process being regulated by W01A8.1 (Chughtai et al., 2015). And our recent experimentation also shows that *C. elegans* embryos lacking W01A8.1 have increased autophagic activity. These experiments have been already published in a Pre-Print article awaiting review (Kaššák et al., 2019). This would be consistent with the role of Perilipins being protectors against lipophagy.

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

Having fairly strong lines of evidence suggesting W01A8.1 as a Perilipin orthologue it was interesting to find the true MED28 orthologue. 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 strong 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 not been truly identified. MED28 has a special position among Mediator subunit proteins for its dual regulatory role, one as a Mediator subunit (Sato et al., 2004; Beyer et al., 2007) and the second, which is cytoplasmic, as a cytoskeletal associated protein (Wiederhold et al., 2004; Lee et al., 2006; Lu et al., 2006; Huang et al., 2012a). One could anticipate that the interaction of cytoplasmic proteins with MED28 may be able to influence gene expression by the translocation of MED28 to the nucleus. A great example that is relatable to the above-mentioned scenario is the beta-catenin protein. The protein acts as an adaptor of interaction between the cytoskeleton and cell adhesion molecules that critically regulates gene expression in the Wnt pathway.

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. In terms of subcellular localisation of F28F8.5 it localised to both nuclear and cytoplasmic compartments in most, if not all, cells throughout development. Interestingly, the phenotypes that we observed in F28F8.5 knock-down and loss of function experiments overlap with the EGFR regulatory cascade in *C. elegans*, especially the developmental defects of the vulva and of male-specific structures, most obviously, 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). This cytoplasmic function of F28F8.5 is supported by the known involvement of the nematode homologue of Grb2, SEM-5, in the regulation of development of male rays. F28F8.5 protein 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***) (Wiederhold et al., 2004). Although, it has to be stressed that there are no close structures available for a high-probability prediction of the structure of F28F8.5. 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)).

Last but not least, 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 a 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 as proposed by Kory et al., (2015). They claim that macromolecular crowding plays a major role in determining LD protein composition. 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. The authors go even further and 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 droplets 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 and is known to promote fusion between droplets, causing the formation of a single droplet per cell (Gong et al., 2011; Jambunathan et al., 2011). Yeast two-hybrid interaction screen revealed the transcription factor NFAT5 (Nuclear factor of activated T cells) is a potential Fsp27 partner of interaction. 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.

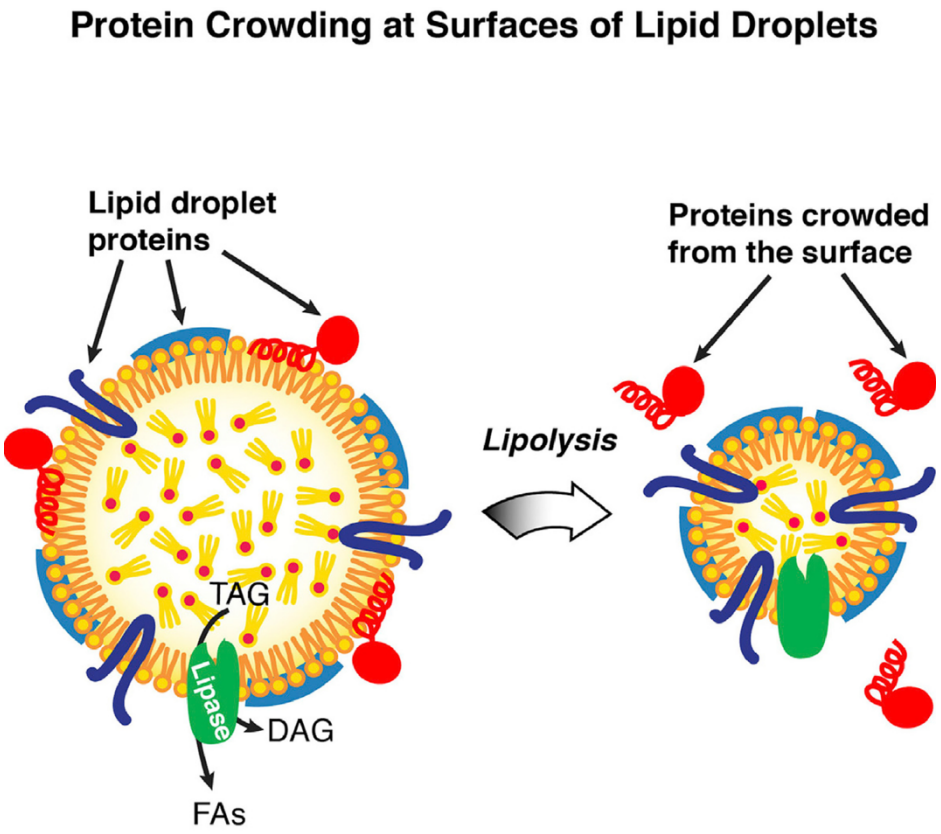


Figure 46

Taken from Kory et al. (2015) with permission. Illustration showing the release of low affinity protein due to LD shrinkage.

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 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 the nucleus. 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, 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, 2018). Although many aspects of the process remain still 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 observations of LDs in the nucleus have been around for a while dating back to the 70's (Hillman and 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. In the last few years, 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 and varied among cell types. 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. The group investigated this and found that various nuclear bodies such as Cajal bodies, speckles, and paraspeckles, which were labelled by antibodies to coilin, SC35, and PSF, respectively, did not exhibit a relationship with nuclear LDs. In contrast, promyelocytic leukaemia (PML) nuclear bodies labelled with anti-PML antibody co-localised with nuclear LDs. They went further and showed that the PML-II protein played a critical role in nuclear LD formation. The function of PML bodies is still a bit of a mystery, but 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 Thé, 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).

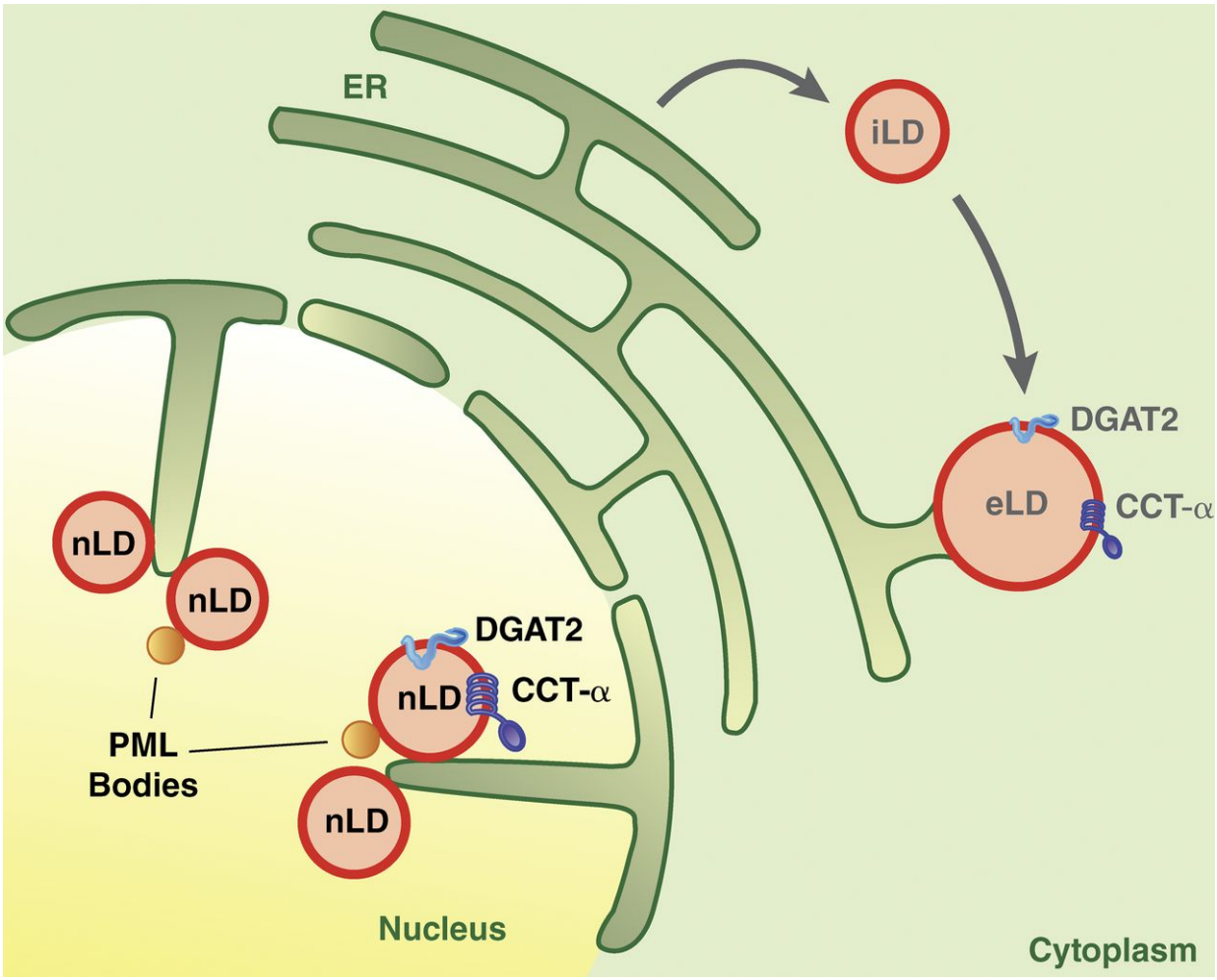


Figure 47

Proposed Model of cytoplasmic and nuclear LDs taken with permission from Farese and Walther, (2016). Initial lipid droplets (iLDs) are formed from the endoplasmic reticulum (ER). A subset of iLDs can be converted to expanding lipid droplets (eLDs) via the establishment of ER-LD membrane bridges and re-localisation of triacylglyceride synthesis enzymes, such as DGAT2, to their surfaces. CTP:phosphocholine cytidylyltransferase α (CCT-α) binds to eLDs with a relative deficiency of phosphatidylcholine (PC) on their surfaces, where it is activated and catalyses PC synthesis. Nuclear LDs form in association with invaginations of the inner nuclear membrane and also are marked by DGAT2 and CCT-α. Nuclear LDs are found in close proximity to PML bodies and may depend on PML proteins for formation.

In a very recent publication of Liu et al., (2018) the authors looked at one of the most commonly found proteins on *C. elegans* LDs (Yang et al., 2012; Zhang et al., 2012; Na et al., 2015). 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 a 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 (Sirtuin 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. And if we 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).

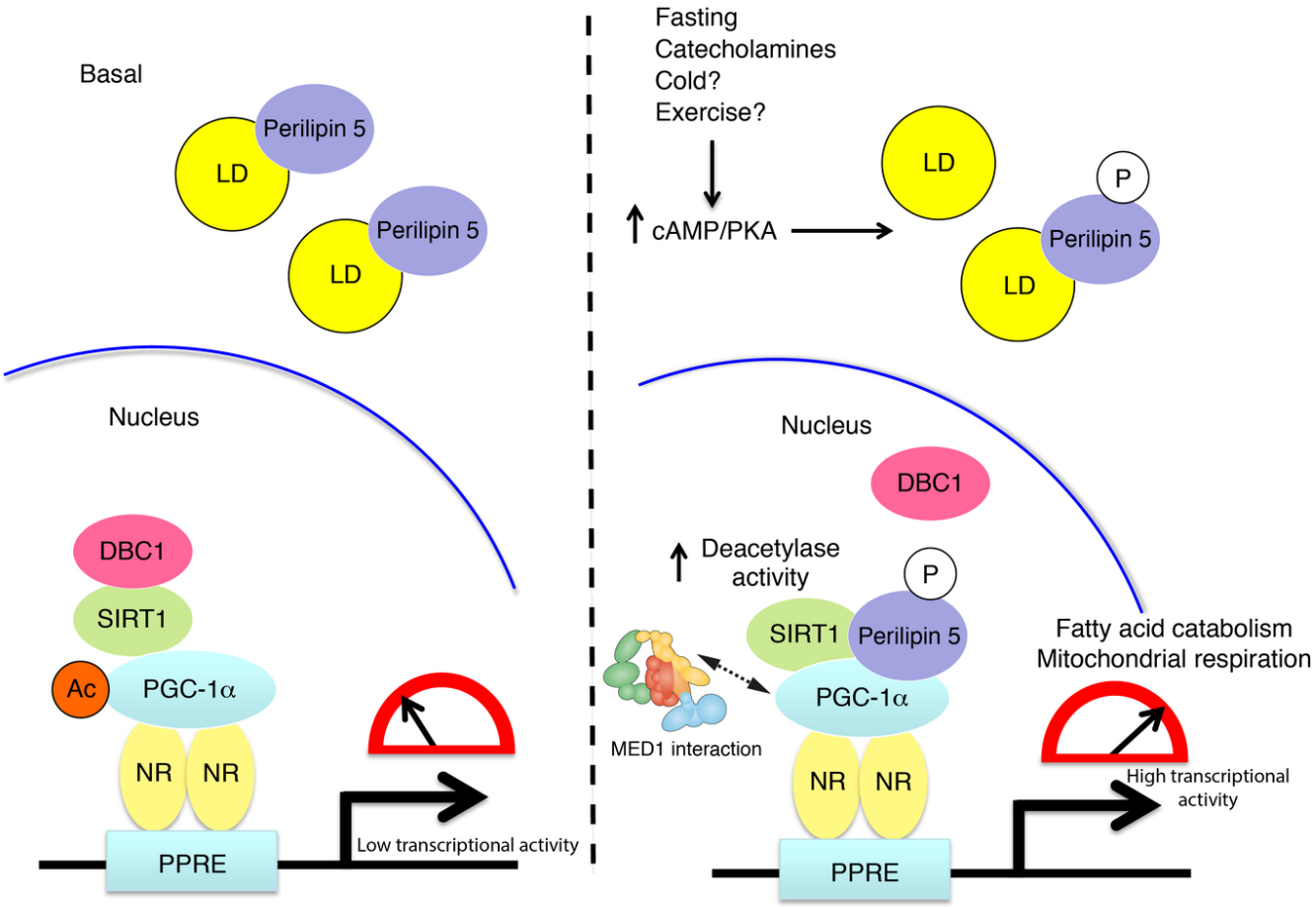


Figure 48

Mechanism of PLIN5 based regulation of gene expression. Adopted under creative common licence from Gallardo-Montejano et al., (2016). PLIN5 under basal conditions is localised only to cytoplasmic LDs and upon PKA based phosphorylation enriches in the nucleus and forms transcriptional complexes with the transcriptional co-regulator PGC-1α. This leads to activation of the deacetylase activity of SIRT1 by displacing DBC1 (Deleted in Breast Cancer-1), an inhibitor of SIRT1, thus promoting PGC-1α activity by deacetylation. PGC-1α also interacts with the Mediator complex via MED1 subunit. By this interaction in the nucleus, a cytoplasmic protein is able to exert control over transcriptional events.

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.

References

Agarwal, V, M Remmert, A Biegert, J Söding. 2008. 'PDBalert: Automatic, Recurrent Remote Homology Tracking and Protein Structure Prediction.'. *BMC Struct Biol* 8: 51.

Ahmadian, M, MJ Abbott, T Tang, CS Hudak, Y Kim, M Bruss, MK Hellerstein, et al. 2011. 'Desnutrin/ATGL Is Regulated by AMPK and Is Required for a Brown Adipose Phenotype.'. *Cell Metab* 13: 739–48.

Ahringer, J. 2006. 'Reverse Genetics.'. *WormBook*. doi:10.1895/wormbook.1.47.1.

Allen, BL, and DJ Taatjes. 2015. 'The Mediator Complex: a Central Integrator of Transcription.'. *Nature Reviews Molecular Cell Biology* 16 (3). Springer Nature: 155–66. doi:10.1038/nrm3951.

Almlöf, T, AE Wallberg, JA Gustafsson, APH Wright. 1998. 'Role of Important Hydrophobic Amino Acids in the Interaction between the Glucocorticoid Receptor.'. tau1-Core Activation Domain and Target Factors'. *Biochemistry* 37 (26). American Chemical Society (ACS): 9586–94. doi:10.1021/bi973029x.

Altschul, SF, TL Madden, AA Schäffer, J Zhang, Z Zhang, W Miller, DJ Lipman. 1997. 'Gapped BLAST and PSI-BLAST: a New Generation of Protein Database Search Programs.'. *Nucleic Acids Res* 25: 3389–3402.

Altun, ZF, and DH Hall. 2009. 'Introduction.'. *WormAtlas*. doi:10.3908/wormatlas.1.1

Alvarez, HM, and A Steinbüchel. 2002. 'Triacylglycerols in Prokaryotic Microorganisms.'. *Appl Microbiol Biotechnol* 60: 367–76.

Anbalagan, M, B Huderson, L Murphy, BG Rowan. 2012. 'Post-Translational Modifications of Nuclear Receptors and Human Disease.'. *Nucl Recept Signal* 10: e001.

Apfeld, J, and C Kenyon. 1999. 'Regulation of Lifespan by Sensory Perception in Caenorhabditis Elegans.'. *Nature* 402: 804–9.

Aramburu, J, K Drews-Elger, A Estrada-Gelonch, J Minguillón, B Morancho, V Santiago, C López-Rodríguez. 2006. 'Regulation of the Hypertonic Stress Response and Other Cellular Functions by the Rel-like Transcription Factor NFAT5.'. *Biochem Pharmacol* 72: 1597–1604.

Arribere, JA, RT Bell, BX Fu, KL Artiles, PS Hartman, AZ Fire. 2014. 'Efficient Marker-Free Recovery of Custom Genetic Modifications with CRISPR/Cas9 in Caenorhabditis Elegans.'. *Genetics* 198: 837–46.

Ashrafi, K, FY Chang, JL Watts, AG Fraser, RS Kamath, J Ahringer, G Ruvkun. 2003. 'Genome-Wide RNAi Analysis of Caenorhabditis Elegans Fat Regulatory Genes.'. *Nature* 421: 268–72.

Ashrafi, K. 2007. 'Obesity and the Regulation of Fat Metabolism.'. *WormBook*, 1–20. doi/10.1895/wormbook.1.130.1

Babonis, LS, and MQ Martindale. 2017. 'Phylogenetic Evidence for the Modular Evolution of Metazoan Signalling Pathways.'. *Philos Trans R Soc Lond B Biol Sci* 372.

Baniahmad, A, AC Köhne, R Renkawitz. 1992. 'A Transferable Silencing Domain Is Present in the Thyroid Hormone Receptor, in the v-ErbA Oncogene Product and in the Retinoic Acid Receptor.'. *EMBO J* 11: 1015–23.

Barry, JB, GM Leong, WB Church, LL Issa, JA Eisman, EM Gardiner. 2003. 'Interactions of SKIP/NCoA-62, TFIIB, and Retinoid X Receptor with Vitamin D Receptor Helix H10 Residues.'. *J Biol Chem* 278: 8224–28.

Bartz, R, WH Li, B Venables, JK Zehmer, MR Roth, R Welti, RG Anderson, P Liu, KD Chapman. 2007. 'Lipidomics Reveals That Adiposomes Store Ether Lipids and Mediate Phospholipid Traffic.'. *J Lipid Res* 48: 837–47.

Baudino, TA, DM Kraichely, SC Jr Jefcoat, SK Winchester, NC Partridge, and PN MacDonald. 1998. 'Isolation and Characterization of a Novel Coactivator Protein, NCoA-62, Involved in Vitamin D-Mediated Transcription.'. *J Biol Chem* 273: 16434–41.

Baugh, LR. 2013. 'To Grow or Not to Grow: Nutritional Control of Development during Caenorhabditis Elegans L1 Arrest.'. *Genetics* 194: 539–55.

Baumli, S, S Hoeppner, P Cramer. 2005. 'A Conserved Mediator Hinge Revealed in the Structure of the MED7·MED21 (Med7·Srb7) Heterodimer.' *Journal of Biological Chemistry* 280 (18). American Society for Biochemistry & Molecular Biology (ASBMB): 18171–78. doi:10.1074/jbc.m413466200.

Beato, M. 2000. 'Steroid Hormone Receptors: an Update'.' *Human Reproduction Update* 6 (3). Oxford University Press (OUP): 225–36. doi:10.1093/humupd/6.3.225.

Belakavadi, M, PK Pandey, R Vijayvargia, JD Fondell. 2008. 'MED1 Phosphorylation Promotes Its Association with Mediator: Implications for Nuclear Receptor Signaling'.' *Molecular and Cellular Biology* 28 (12). American Society for Microbiology: 3932–42. doi:10.1128/mcb.02191-07.

Bell, M, H Wang, H Chen, JC McLenithan, DW Gong, RZ Yang, D Yu, et al. 2008. 'Consequences of Lipid Droplet Coat Protein Downregulation in Liver Cells: Abnormal Lipid Droplet Metabolism and Induction of Insulin Resistance.'. *Diabetes* 57: 2037–45.

Beller, M, AV Bulankina, HH Hsiao, H Urlaub, H Jäckle, and RP Kühnlein. 2010. 'PERILIPIN-Dependent Control of Lipid Droplet Structure and Fat Storage in *Drosophila*.'. *Cell Metab* 12: 521–32.

Beyer, SK, LR Beauchamp, MF Lee, JF Gusella, AM Näär, V Ramesh. 2007. 'Mediator Subunit MED28 (Magicin) Is a Repressor of Smooth Muscle Cell Differentiation'.' *Journal of Biological Chemistry* 282 (44). American Society for Biochemistry & Molecular Biology (ASBMB): 32152–57. doi:10.1074/jbc.m706592200.

Bi, J, Y Xiang, H Chen, Z Liu, S Grönke, RP Kühnlein, X Huang. 2012. 'Opposite and Redundant Roles of the Two *Drosophila* Perilipins in Lipid Mobilization.'. *J Cell Sci* 125: 3568–77.

Bickel, PE, JT Tansey, MA Welte. 2009. 'PAT Proteins, an Ancient Family of Lipid Droplet Proteins That Regulate Cellular Lipid Stores.'. *Biochim Biophys Acta* 1791: 419–40.

Biddie SC, L Gordon, L Hager. 2009. 'Glucocorticoid Receptor Dynamics and Gene Regulation.'.*Stress* 12 (3). Informa UK Limited: 193–205. doi:10.1080/10253890802506409.

Biegert, A, J Söding. 2008. 'De Novo Identification of Highly Diverged Protein Repeats by Probabilistic Consistency.'. *Bioinformatics* 24: 807–14.

Bird, AP. 1986. 'CpG-Rich Islands and the Function of DNA Methylation.'. *Nature* 321: 209–13.

Birney, E, M Clamp, R Durbin. 2004. 'GeneWise and Genomewise.'. *Genome Res* 14: 988–95.

Bonilla, E, and A Prelle. 1987. 'Application of Nile Blue and Nile Red, Two Fluorescent Probes, for Detection of Lipid Droplets in Human Skeletal Muscle.'. *J Histochem Cytochem* 35: 619–21.

Boulant, S, MW Douglas, L Moody, A Budkowska, P Targett-Adams, J McLauchlan. 2008. 'Hepatitis C Virus Core Protein Induces Lipid Droplet Redistribution in a Microtubule- and Dynein-Dependent Manner.'. *Traffic* 9: 1268–82.

Bourbon, HM. 2008. 'Comparative Genomics Supports a Deep Evolutionary Origin for the Large Four-Module Transcriptional Mediator Complex.'. *Nucleic Acids Research* 36 (12). Oxford University Press (OUP): 3993–4008. doi:10.1093/nar/gkn349.

Boyer, G, MED Martin, E Lees, R Ricciardi, AJ Berk. 1999. 'Mammalian Srb/Mediator Complex Is Targeted by Adenovirus E1A Protein.'. *Nature* 399 (6733). Springer Nature: 276–79. doi:10.1038/20466.

Brasaemle, DL, DM Levin, DC Adler-Wailes, C Londos. 2000. 'The Lipolytic Stimulation of 3T3-L1 Adipocytes Promotes the Translocation of Hormone-Sensitive Lipase to the Surfaces of Lipid Storage Droplets.'. *Biochim Biophys Acta* 1483: 251–62.

Brasaemle, DL. 2007. 'Thematic Review Series: Adipocyte Biology. The Perilipin Family of Structural Lipid Droplet Proteins: Stabilization of Lipid Droplets and Control of Lipolysis.'. *J Lipid Res* 48: 2547–59.

Brenner, S. 1974. 'The Genetics of Caenorhabditis Elegans.'. *Genetics* 77: 71–94.

Bridgham, JT, GN Eick, C Larroux, K Deshpande, MJ Harms, ME Gauthier, EA Ortlund, BM Degnan, JW Thornton. 2010. 'Protein Evolution by Molecular Tinkering: Diversification of the Nuclear Receptor Superfamily from a Ligand-Dependent Ancestor.'. *PLoS Biol* 8.

Bulankina, AV, A Deggerich, D Wenzel, K Mutenda, JG Wittmann, MG Rudolph, KN Burger, and S Höning. 2009. 'TIP47 Functions in the Biogenesis of Lipid Droplets.'. *J Cell Biol* 185: 641–55.

Bussell, R, and D Eliezer. 2003. 'A Structural and Functional Role for 11-Mer Repeats in alpha-Synuclein and Other Exchangeable Lipid Binding Proteins.'. *Journal of Molecular Biology* 329 (4). Elsevier BV: 763–78. doi:10.1016/s0022-2836(03)00520-5.

C. elegans Sequencing Consortium. 1998. 'Genome Sequence of the Nematode C. Elegans: a Platform for Investigating Biology.'. *Science* 282: 2012–18.

Cahnmann, HJ 1995. 'A Fast Photoisomerization Method for the Preparation of Tritium-Labeled 9-*cis*-Retinoic Acid of High Specific Activity.'. Analytical Biochemistry 227, 49–53 Elsevier BV.

Carrera, I, F Janody, N Leeds, F Duveau, JE Treisman. 2008. 'Pygopus Activates Wingless Target Gene Transcription through the Mediator Complex Subunits Med12 and Med13.'. *Proceedings of the National Academy of Sciences* 105 (18). Proceedings of the National Academy of Sciences: 6644–49. doi:10.1073/pnas.0709749105.

Cevher, MA, Y Shi, D Li, BT Chait, S Malik, RG Roeder. 2014. 'Reconstitution of Active Human Core Mediator Complex Reveals a Critical Role of the MED14 Subunit.'. *Nature Structural & Molecular Biology* 21 (12). Springer Nature: 1028–34. doi:10.1038/nsmb.2914.

Chakrabarti, P, T English, J Shi, CM Smas, KV Kandror. 2010. 'Mammalian Target of Rapamycin Complex 1 Suppresses Lipolysis, Stimulates Lipogenesis, and Promotes Fat Storage.'. *Diabetes* 59: 775–81.

Chang, CW, D Sud, MA Mycek. 2007. 'Fluorescence Lifetime Imaging Microscopy.'. *Methods Cell Biol* 81: 495–524.

Chen, JD, and RM Evans. 1995. 'A Transcriptional Co-Repressor That Interacts with Nuclear Hormone Receptors.'. *Nature* 377 (6548). Springer Nature: 454–57. doi:10.1038/377454a0.

Chen, W, and RG Roeder. 2011. 'Mediator-Dependent Nuclear Receptor Function.'. *Semin Cell Dev Biol* 22: 749–58.

Chong, BM, TD Russell, J Schaack, DJ Orlicky, P Reigan, M Ladinsky, JL McManaman. 2011. 'The Adipophilin C Terminus Is a Self-Folding Membrane-Binding Domain That Is Important for Milk Lipid Secretion.'. *J Biol Chem* 286: 23254–65.

Chughtai, AA, F Kaššák, M Kostrouchová, JP Novotný, MW Krause, V Saudek, Z Kostrouch, M Kostrouchová. 2015. 'Perilipin-Related Protein Regulates Lipid Metabolism in C. Elegans.'. *PeerJ* 3: e1213.

Cohen, RN, A Putney, FE Wondisford, AN. Hollenberg. 2000. 'The Nuclear Corepressors Recognize Distinct Nuclear Receptor Complexes.'. *Molecular Endocrinology* 14 (6). The Endocrine Society: 900–914. doi:10.1210/mend.14.6.0474.

Cole, NB., DD Murphy, T Grider, S Rueter, D Brasaemle, RL Nussbaum. 2001. 'Lipid Droplet Binding and Oligomerization Properties of the Parkinsons Disease Protein alpha-Synuclein.'. *Journal of Biological Chemistry* 277 (8). American Society for Biochemistry & Molecular Biology (ASBMB): 6344–52. doi:10.1074/jbc.m108414200.

Committee Nuclear Receptors Nomenclature. 1999. 'A unified nomenclature system for the nuclear receptor superfamily.'. Cell 97, 161-3.

Conaway, RC, and JW Conaway. 2011. 'Function and Regulation of the Mediator Complex.'. *Curr Opin Genet Dev* 21: 225–30.

Conaway, RC, and JW Conaway. 2011a. 'Origins and Activity of the Mediator Complex.'. *Seminars in Cell & Developmental Biology* 22 (7). Elsevier BV: 729–34. doi:10.1016/j.semcdb.2011.07.021.

Conaway, RC, S Sato, C Tomomori-Sato, T Yao, JW Conaway. 2005. 'The Mammalian Mediator Complex and Its Role in Transcriptional Regulation.'. *Trends Biochem Sci* 30: 250–55.

Conaway, RC., and JW Conaway. 2013. 'The Mediator Complex and Transcription Elongation.'. *Biochimica Et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1829 (1). Elsevier BV: 69–75. doi:10.1016/j.bbagrm.2012.08.017.

Cosma, MP. 2002. 'Ordered Recruitment: Gene-Specific Mechanism of Transcription Activation.'. *Mol Cell* 10: 227–36.

Costa, M, M Weir, A Coulson, J Sulston, C Kenyon. 1988. 'Posterior Pattern Formation in C. Elegans Involves Position-Specific Expression of a Gene Containing a Homeobox.'. *Cell* 55: 747–56.

Cuervo, AM, and E Wong. 2014. 'Chaperone-Mediated Autophagy: Roles in Disease and Aging.'. *Cell Res* 24: 92–104.

Cuff, JA, and GJ Barton. 2000. 'Application of Multiple Sequence Alignment Profiles to Improve Protein Secondary Structure Prediction.'. *Proteins* 40: 502–11.

Czabany, T, A Wagner, D Zweytick, K Lohner, E Leitner, E Ingolic, G Daum. 2008. 'Structural and Biochemical Properties of Lipid Particles from the Yeast Saccharomyces Cerevisiae.'. *J Biol Chem* 283: 17065–74.

Damm, K, CC Thompson, RM Evans. 1989. 'Protein Encoded by v-ErbA Functions as a Thyroid-Hormone Receptor Antagonist.'. *Nature* 339 (6226). Springer Nature: 593–97. doi:10.1038/339593a0.

Davidson, E. 2001. 'Regulatory Hardwiring: A Brief Overview of the Genomic Control Apparatus and Its Causal Role in Development and Evolution.'. In *Genomic Regulatory Systems*, 1–23. Elsevier. doi:10.1016/b978-012205351-1/50001-x.

Di, TP, S Moretti, I Xenarios, M Orobitg, A Montanyola, JM Chang, JF Taly, C Notredame. 2011. 'T-Coffee: a Web Server for the Multiple Sequence Alignment of Protein and RNA Sequences Using Structural Information and Homology Extension.'. *Nucleic Acids Res* 39: W13–7.

Dickinson, DJ, AM Pani, JK Heppert, CD Higgins, B Goldstein. 2015. 'Streamlined Genome Engineering with a Self-Excising Drug Selection Cassette.'. *Genetics* 200: 1035–49.

Dickinson, DJ, and B Goldstein. 2016. 'CRISPR-Based Methods for Caenorhabditis Elegans Genome Engineering.'. *Genetics* 202: 885–901.

Dickinson, DJ, JD Ward, DJ Reiner, B Goldstein. 2013. 'Engineering the Caenorhabditis Elegans Genome Using Cas9-Triggered Homologous Recombination.'. *Nat Methods* 10: 1028–34.

Ding, Y, Y Wu, R Zeng, and K Liao. 2012. 'Proteomic Profiling of Lipid Droplet-Associated Proteins in Primary Adipocytes of Normal and Obese Mouse.'. *Acta Biochim Biophys Sin (Shanghai)* 44: 394–406.

Dozin, B, HJ Cahnmann, VM Nikodem. 1985. 'Identification of thyroid hormone receptors in rat liver nuclei by photoaffinity labeling with L-thyroxine and triiodo-L-thyronine.'. Biochemistry 24, 5197-202.

Dozin, B, MA Magnuson, VM Nikodem. 1985a. 'Tissue-specific regulation of two functional malic enzyme mRNAs by triiodothyronine.'. Biochemistry 24, 5581-6.

Duncan, RE, M Ahmadian, K Jaworski, E Sarkadi-Nagy, HS Sul. 2007. 'Regulation of Lipolysis in Adipocytes.'. *Annu Rev Nutr* 27: 79–101.

Dupuy, D. 2004. 'A First Version of the Caenorhabditis Elegans Promoterome.'. *Genome Research* 14 (10b). Cold Spring Harbor Laboratory: 2169–75. doi:10.1101/gr.2497604.

Ecsedi, M, M Rausch, H Großhans. 2015. 'The Let-7 MicroRNA Directs Vulval Development through a Single Target.'. *Dev Cell* 32: 335–44.

Egea, PF, BP Klaholz, D Moras. 2000. 'Ligand-protein interactions in nuclear receptors of hormones.'. FEBS Lett 476, 62-7 (2000).

Eitel, M, HJ Osigus, R DeSalle, B Schierwater. 2013. 'Global diversity of the Placozoa'. PLoS One 8, e57131.

Eitel, M, L Guidi, H Hadrys, M Balsamo, B Schierwater. 2011. 'New Insights into Placozoan Sexual Reproduction and Development.'. PLoS ONE 6, e19639 Public Library of Science (PLoS).

Elmlund, H, V Baraznenok, M Lindahl, CO Samuelsen, PJB Koeck, S Holmberg, H Hebert, CM Gustafsson. 2006. 'The Cyclin-Dependent Kinase 8 Module Sterically Blocks Mediator Interactions with RNA Polymerase II.'. *Proceedings of the National Academy of Sciences* 103 (43). Proceedings of the National Academy of Sciences: 15788–93. doi:10.1073/pnas.0607483103.

Esnault, C, Y Ghavi-Helm, S Brun, J Soutourina, N Van Berkum, C Boschiero, F Holstege, M Werner. 2008. 'Mediator-Dependent Recruitment of TFIIH Modules in Preinitiation Complex.'. *Molecular Cell* 31 (3). Elsevier BV: 337–46. doi:10.1016/j.molcel.2008.06.021.

Eyboulet, F, S Wydau-Dematteis, T Eychenne, O Alibert, H Neil, C Boschiero, MC Nevers, et al. 2015. 'Mediator Independently Orchestrates Multiple Steps of Preinitiation Complex Assemblyin Vivo.'. *Nucleic Acids Research* 43 (19). Oxford University Press (OUP): 9214–31. doi:10.1093/nar/gkv782.

Eychenne, T, E Novikova, MB Barrault, O Alibert, C Boschiero, N Peixeiro, D Cornu, et al. 2016. 'Functional Interplay between Mediator and TFIIB in Preinitiation Complex Assembly in Relation to Promoter Architecture.'. *Genes & Development* 30 (18). Cold Spring Harbor Laboratory: 2119–32. doi:10.1101/gad.285775.116.

Fantappié, MR, FM Bastos de Oliveira, R de Moraes Maciel, FD Rumjanek, W Wu, PT LoVerde. 2008. 'Cloning of SmNCoA-62, a Novel Nuclear Receptor Co-Activator from Schistosoma Mansoni: Assembly of a Complex with a SmRXR1/SmNR1 Heterodimer, SmGCN5 and SmCBP1.'. *Int J Parasitol* 38: 1133–47.

Farese, RV Jr, and TC Walther. 2016. 'Lipid Droplets Go Nuclear.'. *J Cell Biol* 212: 7–8.

Feingold, KR, and C Grunfeld. 2000. 'Introduction to Lipids and Lipoproteins.'. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK305896/

Flanagan, PM., RJ Kelleher, MH Sayre, H Tschochner, RD. Kornberg. 1991. 'A Mediator Required for Activation of RNA Polymerase II Transcription in Vitro.'. *Nature* 350 (6317). Springer Nature: 436–38. doi:10.1038/350436a0.

Fondell, JD, H Ge, RG Roeder. 1996. 'Ligand Induction of a Transcriptionally Active Thyroid Hormone Receptor Coactivator Complex.'. *Proceedings of the National Academy of Sciences* 93 (16). Proceedings of the National Academy of Sciences: 8329–33. doi:10.1073/pnas.93.16.8329.

Ford, J, IJ McEwan, APH Wright, JA Gustafsson. 1997. 'Involvement of the Transcription Factor IID Protein Complex in Gene Activation by the N-Terminal Transactivation Domain of the Glucocorticoid Receptorin Vitro.'. *Molecular Endocrinology* 11 (10). The Endocrine Society: 1467–75. doi:10.1210/mend.11.10.9995.

Forman, BM, HH Samuels. 1990.'Dimerization among nuclear hormone receptors.'. New Biol 2, 587-94.

Forman, BM, HH Samuels. 1990a.'Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model.'. Mol Endocrinol 4, 1293-301.

Fowler, SD, and P Greenspan. 1985. 'Application of Nile Red, a Fluorescent Hydrophobic Probe, for the Detection of Neutral Lipid Deposits in Tissue Sections: Comparison with Oil Red O.'. *J Histochem Cytochem* 33: 833–36.

Fowler, SD, WJ Brown, J Warfel, P Greenspan. 1987. 'Use of Nile Red for the Rapid in Situ Quantitation of Lipids on Thin-Layer Chromatograms.'. *J Lipid Res* 28: 1225–32.

Frøkjær-Jensen, C, MW Davis, CE Hopkins, BJ Newman, JM Thummel, SP Olesen, M Grunnet, EM Jorgensen. 2008. 'Single-Copy Insertion of Transgenes in Caenorhabditis Elegans.'. *Nat Genet* 40: 1375–83.

Frøkjær-Jensen, C, MW Davis, G Hollopeter, J Taylor, TW Harris, P Nix, R Lofgren, et al. 2010. 'Targeted Gene Deletions in C. Elegans Using Transposon Excision.'. *Nat Methods* 7: 451–53.

Frøkjær-Jensen, C, MW Davis, M Ailion, EM Jorgensen. 2012. 'Improved Mos1-Mediated Transgenesis in C. Elegans.'. *Nat Methods* 9: 117–18.

Gallardo-Montejano, VI, G Saxena, CM Kusminski, C Yang, JL McAfee, L Hahner, K Hoch, W Dubinsky, VA Narkar, and PE Bickel. 2016. 'Nuclear Perilipin 5 Integrates Lipid Droplet Lipolysis with PGC-1α/SIRT1-Dependent Transcriptional Regulation of Mitochondrial Function.'. *Nat Commun* 7: 12723.

Garcia, A, A Sekowski, V Subramanian, DL Brasaemle. 2002. 'The Central Domain Is Required to Target and Anchor Perilipin A to Lipid Droplets.'. *Journal of Biological Chemistry* 278 (1). American Society for Biochemistry & Molecular Biology (ASBMB): 625–35. doi:10.1074/jbc.m206602200.

Garton, AJ, DG Campbell, D Carling, DG Hardie, RJ Colbran, SJ Yeaman. 1989. 'Phosphorylation of Bovine Hormone-Sensitive Lipase by the AMP-Activated Protein Kinase. A Possible Antilipolytic Mechanism.'. *Eur J Biochem* 179: 249–54.

Ge, K, M Guermah, CX Yuan, M Ito, AE. Wallberg, BM Spiegelman, RG. Roeder. 2002. 'Transcription Coactivator TRAP220 Is Required for PPARγ2-Stimulated Adipogenesis.'. *Nature* 417 (6888). Springer Nature: 563–67. doi:10.1038/417563a.

Ge, K, YW Cho, H Guo, TB Hong, M Guermah, M Ito, H Yu, M Kalkum, RG Roeder. 2007. 'Alternative Mechanisms by Which Mediator Subunit MED1/TRAP220 Regulates Peroxisome Proliferator-Activated Receptor-Stimulated Adipogenesis and Target Gene Expression.'. *Molecular and Cellular Biology* 28 (3). American Society for Microbiology: 1081–91. doi:10.1128/mcb.00967-07.

Georgiadi, A, and S Kersten. 2012. 'Mechanisms of Gene Regulation by Fatty Acids.'. *Adv Nutr* 3: 127–34.

Giguère, V. 1999. 'Orphan Nuclear Receptors: From Gene to Function1.'. *Endocrine Reviews* 20 (5). The Endocrine Society: 689–725. doi:10.1210/edrv.20.5.0378.

Gong, J, Z Sun, L Wu, W Xu, N Schieber, D Xu, G Shui, H Yang, RG Parton, and P Li. 2011. 'Fsp27 Promotes Lipid Droplet Growth by Lipid Exchange and Transfer at Lipid Droplet Contact Sites.'. *J Cell Biol* 195: 953–63.

Granneman, JG, HP Moore, EP Mottillo, Z Zhu, L Zhou. 2011. 'Interactions of Perilipin-5 (Plin5) with Adipose Triglyceride Lipase.'. *J Biol Chem* 286: 5126–35.

Granneman, JG, HP Moore, EP Mottillo, Z Zhu. 2009. 'Functional Interactions between Mldp (LSDP5) and Abhd5 in the Control of Intracellular Lipid Accumulation.'. *J Biol Chem* 284: 3049–57.

Granneman, JG, HP Moore, R Krishnamoorthy, M Rathod. 2009a. 'Perilipin Controls Lipolysis by Regulating the Interactions of AB-Hydrolase Containing 5 (Abhd5) and Adipose Triglyceride Lipase (Atgl).'. *J Biol Chem* 284: 34538–44.

Granneman, JG, HP Moore, RL Granneman, AS Greenberg, MS Obin, Z Zhu. 2007. 'Analysis of Lipolytic Protein Trafficking and Interactions in Adipocytes.'. *J Biol Chem* 282: 5726–35.

Grants, JM, GY Goh, S Taubert. 2015. 'The Mediator Complex of Caenorhabditis Elegans: Insights into the Developmental and Physiological Roles of a Conserved Transcriptional Coregulator.'. *Nucleic Acids Res* 43: 2442–53.

Grants, JM, LT Ying, A Yoda, CC You, H Okano, H Sawa, and S Taubert. 2016. 'The Mediator Kinase Module Restrains Epidermal Growth Factor Receptor Signaling and Represses Vulval Cell Fate Specification in Caenorhabditis Elegans.'. *Genetics* 202: 583–99.

Graupner, G, KN Wills, M Tzukerman, XK Zhang, and M Pfahl. 1989. 'Dual Regulatory Role for Thyroid-Hormone Receptors Allows Control of Retinoic-Acid Receptor Activity.'. *Nature* 340: 653–56.

Greenberg, AS, JJ Egan, SA Wek, NB Garty, EJ Blanchette-Mackie, C Londos. 1991. 'Perilipin, a Major Hormonally Regulated Adipocyte-Specific Phosphoprotein Associated with the Periphery of Lipid Storage Droplets.'. *J Biol Chem* 266: 11341–46.

Greenberg, AS, WJ Shen, K Muliro, S Patel, SC Souza, RA Roth, FB Kraemer. 2001. 'Stimulation of Lipolysis and Hormone-Sensitive Lipase via the Extracellular Signal-Regulated Kinase Pathway.'. *J Biol Chem* 276: 45456–61.

Greenspan, P, EP Mayer, SD Fowler. 1985. 'Nile Red: a Selective Fluorescent Stain for Intracellular Lipid Droplets.'. *J Cell Biol* 100: 965–73.

Grishok, A. 2005. 'RNAi Mechanisms in Caenorhabditis Elegans.'. *FEBS Lett* 579: 5932–39.

Gruber, A, I Cornaciu, A Lass, M Schweiger, M Poeschl, C Eder, M Kumari, et al. 2010. 'The N-Terminal Region of Comparative Gene Identification-58 (CGI-58) Is Important for Lipid Droplet Binding and Activation of Adipose Triglyceride Lipase.'. *J Biol Chem* 285: 12289–98.

Guglielmi, B. 2004. 'A High Resolution Protein Interaction Map of the Yeast Mediator Complex.'. *Nucleic Acids Research* 32 (18). Oxford University Press (OUP): 5379–91. doi:10.1093/nar/gkh878.

Guo, Y, TC Walther, M Rao, N Stuurman, G Goshima, K Terayama, JS Wong, RD Vale, P Walter, RV Farese. 2008. 'Functional Genomic Screen Reveals Genes Involved in Lipid-Droplet Formation and Utilization.'. *Nature* 453: 657–61.

Hager, GL, JG McNally, T Misteli. 2009. 'Transcription Dynamics.'. *Molecular Cell* 35 (6). Elsevier BV: 741–53. doi:10.1016/j.molcel.2009.09.005.

Halanych, KM. 2004. 'Invertebrates: Invertebrate Zoology: A Functional Evolutionary Approach.'. Systematic Biology 53, 662–664 Oxford University Press (OUP).

Hashemi, HF, and JM Goodman. 2015. 'The Life Cycle of Lipid Droplets.'. *Curr Opin Cell Biol* 33: 119–24.

Heery, DM, E Kalkhoven, S Hoare, MG Parker. 1997. 'A Signature Motif in Transcriptional Co-Activators Mediates Binding to Nuclear Receptors.'. *Nature* 387 (6634). Springer Nature: 733–36. doi:10.1038/42750.

Henriksson, A, T Almlöf, J Ford, I J McEwan, JA Gustafsson, and AP Wright. 1997. 'Role of the Ada Adaptor Complex in Gene Activation by the Glucocorticoid Receptor.'. *Molecular and Cellular Biology* 17 (6). American Society for Microbiology: 3065–73. doi:10.1128/mcb.17.6.3065.

Hermanson, O, CK Glass, MG Rosenfeld. 2002. 'Nuclear Receptor Coregulators: Multiple Modes of Modification.'. *Trends in Endocrinology & Metabolism* 13 (2). Elsevier BV: 55–60. doi:10.1016/s1043-2760(01)00527-6.

Hickenbottom, SJ, AR Kimmel, C Londos, JH Hurley. 2004. 'Structure of a Lipid Droplet Protein.'. *Structure* 12 (7). Elsevier BV: 1199–1207. doi:10.1016/j.str.2004.04.021.

Hillman, N, and R Hillman. 1975. 'Ultrastructural Studies of tw32/tw32 Mouse Embryos.'. *J Embryol Exp Morphol* 33: 685–95.

Hilser, VJ, and EB Thompson. 2011. 'Structural Dynamics, Intrinsic Disorder, and Allostery in Nuclear Receptors as Transcription Factors.'. *J Biol Chem* 286: 39675–82.

Hittelman, AB. 1999. 'Differential Regulation of Glucocorticoid Receptor Transcriptional Activation via AF-1-Associated Proteins.'. *The EMBO Journal* 18 (19). Wiley-Blackwell: 5380–88. doi:10.1093/emboj/18.19.5380.

Holm, C. 2003. 'Molecular Mechanisms Regulating Hormone-Sensitive Lipase and Lipolysis.'. *Biochem Soc Trans* 31: 1120–24.

Holstege, FCP, EG Jennings, JJ Wyrick, TI Lee, CJ Hengartner, MR Green, TR Golub, ES Lander, RA Young. 1998. 'Dissecting the Regulatory Circuitry of a Eukaryotic Genome.'. *Cell* 95 (5). Elsevier BV: 717–28. doi:10.1016/s0092-8674(00)81641-4.

Hörlein, AJ, AM Näär, T Heinzel, J Torchia, B Gloss, R Kurokawa, A Ryan, et al. 1995. 'Ligand-Independent Repression by the Thyroid Hormone Receptor Mediated by a Nuclear Receptor Co-Repressor.'. *Nature* 377 (6548). Springer Nature: 397–404. doi:10.1038/377397a0.

Hsu, PD, ES Lander, F Zhang. 2014. 'Development and Applications of CRISPR-Cas9 for Genome Engineering.'. *Cell* 157: 1262–78.

Hu, PJ, J Xu, G Ruvkun. 2006. 'Two Membrane-Associated Tyrosine Phosphatase Homologs Potentiate C. Elegans AKT-1/PKB Signaling.'. *PLoS Genet* 2: e99.

Hu, X, and MA Lazar. 1999. 'The CoRNR Motif Controls the Recruitment of Corepressors by Nuclear Hormone Receptors.'. *Nature* 402 (6757). Springer Nature: 93–96. doi:10.1038/47069.

Hu, X, S Malik, CC Negroiu, K Hubbard, CN Velalar, B Hampton, D Grosu, J Catalano, RG Roeder, A Gnatt. 2006a. 'A Mediator-Responsive Form of Metazoan RNA Polymerase II.'. *Proceedings of the National Academy of Sciences* 103 (25). Proceedings of the National Academy of Sciences: 9506–11. doi:10.1073/pnas.0603702103.

Huang, CY, YH Chou, NT Hsieh, HH Chen, MF Lee. 2012a. 'MED28 Regulates MEK1-Dependent Cellular Migration in Human Breast Cancer Cells'. *Journal of Cellular Physiology* 227 (12). Wiley-Blackwell: 3820–27. doi:10.1002/jcp.24093.

Huang, S, M Hölzel, T Knijnenburg, A Schlicker, P Roepman, U McDermott, M Garnett, et al. 2012. 'MED12 Controls the Response to Multiple Cancer Drugs through Regulation of TGF-β Receptor Signaling.'. *Cell* 151: 937–50.

Imamoto, F. 1973. 'Diversity of Regulation of Genetic Transcription.'. *Journal of Molecular Biology* 74 (2). Elsevier BV: 113–36. doi:10.1016/0022-2836(73)90102-2.

Inoue, T, M Ailion, S Poon, HK Kim, JH Thomas, PW Sternberg. 2007. 'Genetic Analysis of Dauer Formation in Caenorhabditis Briggsae.'. *Genetics* 177: 809–18.

International Human Genome Sequencing Consortium. 2004. 'Finishing the Euchromatic Sequence of the Human Genome.'. *Nature* 431: 931–45.

Itabe, H, T Yamaguchi, S Nimura, N Sasabe. 2017. 'Perilipins: a Diversity of Intracellular Lipid Droplet Proteins.'. *Lipids in Health and Disease* 16 (1). Springer Nature. doi:10.1186/s12944-017-0473-y.

Ito, M CX Yuan, HJ Okano, RB Darnell, RG Roeder. 2000. 'Involvement of the TRAP220 Component of the TRAP/SMCC Coactivator Complex in Embryonic Development and Thyroid Hormone Action.'. *Molecular Cell* 5 (4). Elsevier BV: 683–93. doi:10.1016/s1097-2765(00)80247-6.

Jackson, AM, and LW Buss. 'Shiny spheres of placozoans (Trichoplax) function in anti-predator defense.'. 2009. Invertebrate Biology 128, 205–212 Wiley.

Jakob, W, S Sagasser, S Dellaporta, P Holland, K Kuhn, B Schierwater. 2004.'The Trox-2 Hox/ParaHox gene of Trichoplax (Placozoa) marks an epithelial boundary.'. Dev Genes Evol 214, 170-5

Jambunathan, S, J Yin, W Khan, Y Tamori, V Puri. 2011. 'FSP27 Promotes Lipid Droplet Clustering and Then Fusion to Regulate Triglyceride Accumulation.'. *PLoS One* 6: e28614.

Jenuwein, T, and CD Allis. 2001. 'Translating the Histone Code.'. *Science* 293: 1074–80.

Jia, Y, N Viswakarma, JK Reddy. 2014. 'Med1 Subunit of the Mediator Complex in Nuclear Receptor-Regulated Energy Metabolism, Liver Regeneration, and Hepatocarcinogenesis.'. *Gene Expr* 16: 63–75.

Jinek, M, K Chylinski, I Fonfara, M Hauer, JA Doudna, and E Charpentier. 2012. 'A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity.'. *Science* 337: 816–21.

Jishage, M, S Malik, U Wagner, B Uberheide, Y Ishihama, X Hu, BT Chait, A Gnatt, B Ren, RG Roeder. 2012. 'Transcriptional Regulation by Pol II(G) Involving Mediator and Competitive Interactions of Gdown1 and TFIIF with Pol II.'. *Molecular Cell* 45 (1). Elsevier BV: 51–63. doi:10.1016/j.molcel.2011.12.014.

Johnson, KM. 2002. 'TFIID and Human Mediator Coactivator Complexes Assemble Cooperatively on Promoter DNA.'. *Genes & Development* 16 (14). Cold Spring Harbor Laboratory: 1852–63. doi:10.1101/gad.995702.

Jolivet, P, L Aymé, A Giuliani, F Wien, T Chardot, Y Gohon. 2017. 'Structural Proteomics: Topology and Relative Accessibility of Plant Lipid Droplet Associated Proteins.'. *J Proteomics* 169: 87–98.

Jones, DT. 1999. 'Protein Secondary Structure Prediction Based on Position-Specific Scoring Matrices.'. *J Mol Biol* 292: 195–202.

Kana R, E Kotabova, R Sobotka, O Prasil. 2012. 'Non-photochemical quenching in cryptophyte alga Rhodomonas salina is located in chlorophyll a/c antennae.'. PLOS ONE 7:e297.

Kana R, EKotabova, M Lukes, S Papacek, CMatonoha, LN Liu, O Prasil, CW Mullineaux. 2014. 'Phycobilisome mobility and its role in the regulation of light harvesting in red algae.'. Plant Physiology 165:1618-1631.

Kang, YK, M Guermah, CX Yuan, RG Roeder. 2002. 'The TRAP/Mediator Coactivator Complex Interacts Directly with Estrogen Receptors and through the TRAP220 Subunit and Directly Enhances Estrogen Receptor Function in Vitro.'. *Proceedings of the National Academy of Sciences* 99 (5). Proceedings of the National Academy of Sciences: 2642–47. doi:10.1073/pnas.261715899.

Kanshin, E, S Wang, L Ashmarina, M Fedjaev, I Nifant'ev, GA Mitchell, AV Pshezhetsky. 2009. 'The Stoichiometry of Protein Phosphorylation in Adipocyte Lipid Droplets: Analysis by N-Terminal Isotope Tagging and Enzymatic Dephosphorylation.'. *Proteomics* 9: 5067–77.

Kaššák, F, AA Chughtai, M Kostrouchová. 2019. 'Evolutionarily Conserved Roles of Caenorhabditis Elegans Perilipin in Lipolysis.'. *PeerJ Preprints*, January. PeerJ. doi:10.7287/peerj.preprints.27467v1.

Kato, Y, R Habas, Y Katsuyama, AM Näär, X He. 2002. 'A Component of the ARC/Mediator Complex Required for TGFβ/Nodal Signalling.'. *Nature* 418 (6898). Springer Nature: 641–46. doi:10.1038/nature00969.

Kaushik, S, and AM Cuervo. 2015. 'Degradation of Lipid Droplet-Associated Proteins by Chaperone-Mediated Autophagy Facilitates Lipolysis.'. *Nat Cell Biol* 17: 759–70.

Kaushik, S, and AM Cuervo. 2016. 'AMPK-Dependent Phosphorylation of Lipid Droplet Protein PLIN2 Triggers Its Degradation by CMA.'. *Autophagy* 12: 432–38.

Kelleher, RJ, PM Flanagan, RD Kornberg. 1990. 'A Novel Mediator between Activator Proteins and the RNA Polymerase II Transcription Apparatus.'. *Cell* 61 (7). Elsevier BV: 1209–15. doi:10.1016/0092-8674(90)90685-8.

Kershaw, EE, M Schupp, HP Guan, NP Gardner, MA Lazar, and JS Flier. 2007. 'PPARgamma Regulates Adipose Triglyceride Lipase in Adipocytes in Vitro and in Vivo.'. *Am J Physiol Endocrinol Metab* 293: E1736–45.

Khan, SA, EE Wollaston-Hayden, TW Markowski, L Higgins, DG Mashek. 2015. 'Quantitative Analysis of the Murine Lipid Droplet-Associated Proteome during Diet-Induced Hepatic Steatosis.'. *J Lipid Res* 56: 2260–72.

Khorasanizadeh, S. 2004. 'The Nucleosome.'. *Cell* 116 (2). Elsevier BV: 259–72. doi:10.1016/s0092-8674(04)00044-3.

Kim, JY, K Tillison, JH Lee, DA Rearick, CM Smas. 2006. 'The Adipose Tissue Triglyceride Lipase ATGL/PNPLA2 Is Downregulated by Insulin and TNF-Alpha in 3T3-L1 Adipocytes and Is a Target for Transactivation by PPARgamma.'. *Am J Physiol Endocrinol Metab* 291: E115–27.

Kim, MY, SJ Hsiao, WL Kraus. 2001. 'A Role for Coactivators and Histone Acetylation in Estrogen Receptor Alpha-Mediated Transcription Initiation.'. *EMBO J* 20: 6084–94.

Kim, S, X Xu, A Hecht, TG Boyer. 2006a. 'Mediator Is a Transducer of Wnt/Beta-Catenin Signaling.'. *Journal of Biological Chemistry* 281 (20). American Society for Biochemistry & Molecular Biology (ASBMB): 14066–75. doi:10.1074/jbc.m602696200.

Kim, SJ, T Tang, M Abbott, JA Viscarra, Y Wang, HS Sul. 2016. 'AMPK Phosphorylates Desnutrin/ATGL and Hormone-Sensitive Lipase To Regulate Lipolysis and Fatty Acid Oxidation within Adipose Tissue.'. *Mol Cell Biol* 36: 1961–76.

Kim, YJ, S Björklund, Y Li, MH Sayre, RD Kornberg. 1994. 'A Multiprotein Mediator of Transcriptional Activation and Its Interaction with the C-Terminal Repeat Domain of RNA Polymerase II.'. *Cell* 77 (4). Elsevier BV: 599–608. doi:10.1016/0092-8674(94)90221-6.

Kimmel, AR, DL Brasaemle, M McAndrews-Hill, C Sztalryd, C Londos. 2010. 'Adoption of PERILIPIN as a Unifying Nomenclature for the Mammalian PAT-Family of Intracellular Lipid Storage Droplet Proteins.'. *J Lipid Res* 51: 468–71.

King-Jones, K, and CS Thummel. 2005. 'Nuclear Receptors–a Perspective from *Drosophila*.'. *Nat Rev Genet* 6: 311–23.

Klose, RJ, and AP Bird. 2006. ‘Genomic DNA Methylation: the Mark and Its Mediators.’. Trends Biochem Sci 31: 89–97.

Knuesel, MT, KD Meyer, C Bernecky, DJ Taatjes. 2009. ‘The Human CDK8 Subcomplex Is a Molecular Switch That Controls Mediator Coactivator Function.’. *Genes & Development* 23 (4). Cold Spring Harbor Laboratory: 439–51. doi:10.1101/gad.1767009.

Kolkhof, P, M Werthebach, A de Venn van, G Poschmann, L Chen, M Welte, K Stühler, M Beller. 2017. ‘A Luciferase-Fragment Complementation Assay to Detect Lipid Droplet-Associated Protein-Protein Interactions.’. *Mol Cell Proteomics* 16: 329–45.

Kornberg, RD. 2007. ‘The Molecular Basis of Eukaryotic Transcription.’. *Proceedings of the National Academy of Sciences* 104 (32). Proceedings of the National Academy of Sciences: 12955–61. doi:10.1073/pnas.0704138104.

Kory, N, AR Thiam, RV Jr Farese, TC Walther. 2015. ‘Protein Crowding Is a Determinant of Lipid Droplet Protein Composition.’. *Dev Cell* 34: 351–63.

Kostrouch, D, M Kostrouchová, P Yilma, AA Chughtai, JP Novotný, P Novák, V Kostrouchová, M Kostrouchová, Z Kostrouch. 2014. ‘SKIP and BIR-1/Survivin Have Potential to Integrate Proteome Status with Gene Expression.’. *J Proteomics* 110: 93–106.

Kostrouch, Z, M Kostrouchova, JE Rall. 1995. ‘Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*.’. Proceedings of the National Academy of Sciences 92, 156–159.

Kostrouchova, M, D Housa, Z Kostrouch, V Saudek, JE Rall. 2002. ‘SKIP Is an Indispensable Factor for Caenorhabditis Elegans Development.’. *Proc Natl Acad Sci U S A* 99: 9254–59.

Kostrouchová, M, D Kostrouch, AA Chughtai, F Kaššák, JP Novotný, V Kostrouchová, A Benda, et al. 2017. ‘The Nematode Homologue of Mediator Complex Subunit 28, F28F8.5, Is a Critical Regulator of C. Elegans Development.’. *PeerJ* 5: e3390.

Kostrouchova, M, Z Kostrouch, V Saudek, J Piatigorsky, JE Rall. 2003. ‘BIR-1, a Caenorhabditis Elegans Homologue of Survivin, Regulates Transcription and Development.’. *Proc Natl Acad Sci U S A* 100: 5240–45.

Kostrouchova, M, Z Kostrouch. 2015. ‘Nuclear receptors in nematode development: Natural experiments made by a phylum.’. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1849, 224–237 Elsevier BV.

Kozusko, K, V Tsang, W Bottomley, YH Cho, S Gandotra, ML Mimmack, K Lim, et al. 2015. ‘Clinical and Molecular Characterization of a Novel PLIN1 Frameshift Mutation Identified in Patients with Familial Partial Lipodystrophy.’. *Diabetes* 64: 299–310.

Krahmer, N, Y Guo, F Wilfling, M Hilger, S Lingrell, K Heger, HW Newman, et al. 2011. ‘Phosphatidylcholine Synthesis for Lipid Droplet Expansion is Mediated by Localized Activation of CTP:Phosphocholine Cytidylyltransferase.’. *Cell Metab* 14: 504–15.

Kralisch, S, J Klein, U Lossner, M Bluher, R Paschke, M Stumvoll, M Fasshauer. 2005. ‘Isoproterenol, TNFalpha, and Insulin Downregulate Adipose Triglyceride Lipase in 3T3-L1 Adipocytes.’. *Mol Cell Endocrinol* 240: 43–49.

Kratochwil, CF, and A Meyer. 2014. ‘Closing the Genotype-Phenotype Gap: Emerging Technologies for Evolutionary Genetics in Ecological Model Vertebrate Systems.’. *BioEssays* 37 (2). Wiley-Blackwell: 213–26. doi:10.1002/bies.201400142.

Kraus, WL, and J Wong. 2002. ‘Nuclear Receptor-Dependent Transcription with Chromatin.’. *European Journal of Biochemistry* 269 (9). Wiley-Blackwell: 2275–83. doi:10.1046/j.1432-1033.2002.02889.x.

Krintel, C, P Osmark, MR Larsen, S Resjö, DT Logan, C Holm. 2008. ‘Ser649 And Ser650 Are the Major Determinants of Protein Kinase A-Mediated Activation of Human Hormone-Sensitive Lipase against Lipid Substrates.’. *PLoS One* 3: e3756.

Krust, A, S Green, P Argos, V Kumar, P Walter, JM Bornert, P Chambon. 1986. 'The Chicken Oestrogen Receptor Sequence: Homology with v-ErbA and the Human Oestrogen and Glucocorticoid Receptors.'. *EMBO J* 5: 891–97.

Kuuluvainen, E, H Hakala, E Havula, MS Estimé, M Rämet, V Hietakangas, TP Mäkelä. 2014. 'Cyclin-Dependent Kinase 8 Module Expression Profiling Reveals Requirement of Mediator Subunits 12 and 13 for Transcription of Serpent-Dependent Innate Immunity Genes In *Drosophila.*'. *Journal of Biological Chemistry* 289 (23). American Society for Biochemistry & Molecular Biology (ASBMB): 16252–61. doi:10.1074/jbc.m113.541904.

Lafontan, M, and D Langin. 2009. 'Lipolysis and Lipid Mobilization in Human Adipose Tissue.'. *Prog Lipid Res* 48: 275–97.

Lai, F, UA Orom, M Cesaroni, M Beringer, DJ Taatjes, GA Blobel, R Shiekhattar. 2013. 'Activating RNAs Associate with Mediator to Enhance Chromatin Architecture and Transcription.'. *Nature* 494 (7438). Springer Nature: 497–501. doi:10.1038/nature11884.

Lallemand-Breitenbach, V, and H de Thé. 2010. 'PML Nuclear Bodies.'. *Cold Spring Harb Perspect Biol* 2: a000661.

Lanz, RB, NJ McKenna, SA Onate, U Albrecht, J Wong, SY Tsai, MJ Tsai, BW O'Malley. 1999. 'A Steroid Receptor Coactivator, SRA, Functions as an RNA and Is Present in an SRC-1 Complex.'. *Cell* 97: 17–27.

Larivière, L, S Geiger, S Hoeppner, S Röther, K Sträßer, P Cramer. 2006. 'Structure and TBP Binding of the Mediator Head Subcomplex Med8Med18Med20.'. *Nature Structural & Molecular Biology* 13 (10). Springer Nature: 895–901. doi:10.1038/nsmb1143.

Lass, A, R Zimmermann, G Haemmerle, M Riederer, G Schoiswohl, M Schweiger, P Kienesberger, JG Strauss, G Gorkiewicz, R Zechner. 2006. 'Adipose Triglyceride Lipase-Mediated Lipolysis of Cellular Fat Stores Is Activated by CGI-58 and Defective in Chanarin-Dorfman Syndrome.'. *Cell Metab* 3: 309–19.

Lass, A, R Zimmermann, M Oberer, R Zechner. 2011. 'Lipolysis - a Highly Regulated Multi-Enzyme Complex Mediates the Catabolism of Cellular Fat Stores.'. *Prog Lipid Res* 50: 14–27.

Latchman, DS. 2004. 'Families of DNA Binding Transcription Factors.'. In *Eukaryotic Transcription Factors*, 77–133. Elsevier. doi:10.1016/b978-012437178-1/50010-2.

Latchman, DS. 2004. 1997. 'Transcription Factors: An Overview.'. *The International Journal of Biochemistry & Cell Biology* 29 (12). Elsevier BV: 1305–12. doi:10.1016/s1357-2725(97)00085-x.

Laudet, V, and H Gronemeyer. 2002. 'DNA Recognition by Nuclear Receptors.'. In *The Nuclear Receptor FactsBook*, 22–36. Elsevier. doi:10.1016/b978-012437735-6/50004-7.

Laudet, V, and H Gronemeyer. 2002a. 'General Organization of Nuclear Receptors.'. In *The Nuclear Receptor FactsBook*, 3–21. Elsevier. doi:10.1016/b978-012437735-6/50003-5.

Laudet, V. 1997. 'Evolution of the Nuclear Receptor Superfamily: Early Diversification from an Ancestral Orphan Receptor.'. *J Mol Endocrinol* 19: 207–26.

Layerenza, JP, P González, MM de Bravo García, MP Polo, MS Sisti, A Ves-Losada. 2013. 'Nuclear Lipid Droplets: a Novel Nuclear Domain.'. *Biochim Biophys Acta* 1831: 327–40.

le Maire, A, C Teyssier, C Erb, M Grimaldi, S Alvarez, AR de Lera, P Balaguer, et al. 2010. 'A Unique Secondary-Structure Switch Controls Constitutive Gene Repression by Retinoic Acid Receptor.'. *Nature Structural & Molecular Biology* 17 (7). Springer Nature: 801–7. doi:10.1038/nsmb.1855.

Lee, JH, J Kong, JY Jang, JS Han, Y Ji, J Lee, JB Kim. 2014. 'Lipid Droplet Protein LID-1 Mediates ATGL-1-Dependent Lipolysis during Fasting in Caenorhabditis Elegans.'. *Mol Cell Biol* 34: 4165–76.

Lee, MF, LR Beauchamp, KS Beyer, JF Gusella, V Ramesh. 2006. 'Magicin Associates with the Src-Family Kinases and Is Phosphorylated upon CD3 Stimulation.'. *Biochemical and Biophysical Research Communications* 348 (3). Elsevier BV: 826–31. doi:10.1016/j.bbrc.2006.07.126.

Lehner, B, C Crombie, J Tischler, A Fortunato, AG Fraser. 2006. 'Systematic Mapping of Genetic Interactions in Caenorhabditis Elegans Identifies Common Modifiers of Diverse Signaling Pathways.'. *Nat Genet* 38: 896–903.

Leong, GM, N Subramaniam, J Figueroa, JL Flanagan, MJ Hayman, JA Eisman, AP Kouzmenko. 2001. 'Ski-Interacting Protein Interacts with Smad Proteins to Augment Transforming Growth Factor-Beta-Dependent Transcription.'. *J Biol Chem* 276: 18243–48.

Leong, GM, N Subramaniam, LL Issa, JB Barry, T Kino, PH Driggers, MJ Hayman, JA Eisman, EM Gardiner. 2004. 'Ski-Interacting Protein, a Bifunctional Nuclear Receptor Coregulator That Interacts with N-CoR/SMRT and p300.'. *Biochem Biophys Res Commun* 315: 1070–76.

Letunic, I, T Doerks, P Bork. 2014. 'SMART: recent updates new developments and status in 2015.'. Nucleic Acids Research 43, D257–D260 Oxford University Press (OUP).

Levine, M, and R Tjian. 2003. 'Transcription Regulation and Animal Diversity.'. *Nature* 424 (6945). Springer Nature: 147–51. doi:10.1038/nature01763.

Li, F, G Ambrosini, EY Chu, J Plescia, S Tognin, PC Marchisio, DC Altieri. 1998. 'Control of Apoptosis and Mitotic Spindle Checkpoint by Survivin.'. *Nature* 396: 580–84.

Li, Z, K Thiel, PJ Thul, M Beller, RP Kühnlein, MA Welte. 2012. 'Lipid Droplets Control the Maternal Histone Supply of *Drosophila* Embryos.'. *Curr Biol* 22: 2104–13.

Libý, P, M Pohludka, J Vohánka, M Kostrouchová, D Kostrouch, M Kostrouchová, JE Rall, Z Kostrouch. 2006. 'BIR-1, the Homologue of Human Survivin, Regulates Expression of Developmentally Active Collagen Genes in C. Elegans.'. *Folia Biol (Praha)* 52: 101–8.

Listenberger, LL, AG Ostermeyer-Fay, EB Goldberg, WJ Brown, DA Brown. 2007. 'Adipocyte Differentiation-Related Protein Reduces the Lipid Droplet Association of Adipose Triglyceride Lipase and Slows Triacylglycerol Turnover.'. *J Lipid Res* 48: 2751–61.

Liu, C, L Zhang, ZM Shao, P Beatty, M Sartippour, TF Lane, SH Barsky, E Livingston, M Nguyen. 2002. 'Identification of a Novel Endothelial-Derived Gene EG-1.'. *Biochemical and Biophysical Research Communications* 290 (1). Elsevier BV: 602–12. doi:10.1006/bbrc.2001.6119.

Liu, Y, S Xu, C Zhang, X Zhu, MA Hammad, X Zhang, M Christian, H Zhang, P Liu. 2018. 'Hydroxysteroid Dehydrogenase Family Proteins on Lipid Droplets through Bacteria, C. Elegans, and Mammals.'. *Biochim Biophys Acta Mol Cell Biol Lipids* 1863: 881–94.

Liu, Z, X Li, Q Ge, M Ding, X Huang. 2014. 'A Lipid Droplet-Associated GFP Reporter-Based Screen Identifies New Fat Storage Regulators in C. Elegans.'. *J Genet Genomics* 41: 305–13.

Long, YC, and JR Zierath. 2006. 'AMP-Activated Protein Kinase Signaling in Metabolic Regulation.'. *J Clin Invest* 116: 1776–83.

Lu, M, L Zhang, M Sartippour, A Norris, M Brooks. 2006. 'EG-1 Interacts with c-Src and Activates Its Signaling Pathway.'. *International Journal of Oncology*, October. Spandidos Publications. doi:10.3892/ijo.29.4.1013.

Ly, K, SJ Reid, RG Snell. 2015. 'Rapid RNA Analysis of Individual Caenorhabditis Elegans.'. *MethodsX* 2: 59–63.

Maglich, JM, A Sluder, X Guan, Y Shi, DD McKee, K Carrick, K Kamdar, TM Willson, JT Moore. 2001. 'Comparison of Complete Nuclear Receptor Sets from the Human, *Caenorhabditis elegans* and *Drosophila* Genomes.'. *Genome Biology* 2 (8). Springer Nature: research0029.1. doi:10.1186/gb-2001-2-8-research0029.

Mak, HY. 2012. 'Lipid Droplets as Fat Storage Organelles in Caenorhabditis Elegans: Thematic Review Series: Lipid Droplet Synthesis and Metabolism: from Yeast to Man.'. *J Lipid Res* 53: 28–33.

Malik, S, AE Wallberg, YK Kang, RG Roeder. 2002. 'TRAP/SMCC/Mediator-Dependent Transcriptional Activation from DNA and Chromatin Templates by Orphan Nuclear Receptor Hepatocyte Nuclear Factor 4.'.

Molecular and Cellular Biology 22 (15). American Society for Microbiology: 5626–37. doi:10.1128/mcb.22.15.5626-5637.2002.

Malik, S, and RG Roeder. 2010. 'The Metazoan Mediator Co-Activator Complex as an Integrative Hub for Transcriptional Regulation.'. *Nature Reviews Genetics* 11 (11). Springer Nature: 761–72. doi:10.1038/nrg2901.

Malik, S, M Guermah, CX Yuan, W Wu, S Yamamura, RG Roeder. 2004. 'Structural and Functional Organization of TRAP220 the TRAP/Mediator Subunit That Is Targeted by Nuclear Receptors.'. *Molecular and Cellular Biology* 24 (18). American Society for Microbiology: 8244–54. doi:10.1128/mcb.24.18.8244-8254.2004.

Malik, S. 2016. 'Eukaryotic Transcription Regulation: Getting to the Heart of the Matter: Commentary on Mediator Architecture and RNA Polymerase II Function by Plaschka Et Al.'. *Journal of Molecular Biology* 428 (12). Elsevier BV: 2575–80. doi:10.1016/j.jmb.2016.04.001.

Malovannaya, A, RB Lanz, SY Jung, Y Bulynko, NT Le, DW Chan, C Ding, et al. 2011. 'Analysis of the Human Endogenous Coregulator Complexome.'. *Cell* 145: 787–99.

Mangelsdorf, DJ, C Thummel, M Beato, P Herrlich, G Schütz, K Umesono, B Blumberg, et al. 1995. 'The Nuclear Receptor Superfamily: the Second Decade.'. *Cell* 83: 835–39.

Martinelli, C, J Spring. 2004. 'Expression pattern of the homeobox gene Not in the basal metazoan *Trichoplax adhaerens*.'. Gene Expr Patterns 4, 443-7 (2004).

Martínez-Redondo, V, AT Pettersson, JL Ruas. 2015. 'The Hitchhiker's Guide to PGC-1α Isoform Structure and Biological Functions.'. *Diabetologia* 58: 1969–77.

Matus, DQ, XY Li, S Durbin, D Agarwal, Q Chi, SJ Weiss, DR Sherwood. 2010. 'In Vivo Identification of Regulators of Cell Invasion across Basement Membranes.'. *Sci Signal* 3: ra35.

McGuffin, LJ, K Bryson, DT Jones. 2000. 'The PSIPRED Protein Structure Prediction Server.'. *Bioinformatics* 16: 404–5.

Meyer, KD, AJ Donner, MT Knuesel, AG York, JM Espinosa, DJ Taatjes. 2008. 'Cooperative Activity of cdk8 and GCN5L within Mediator Directs Tandem Phosphoacetylation of Histone H3.'. *The EMBO Journal*, April. Wiley-Blackwell. doi:10.1038/emboj.2008.78.

Misteli, T. 2001. 'Protein Dynamics: Implications for Nuclear Architecture and Gene Expression.'. *Science* 291 (5505). American Association for the Advancement of Science (AAAS): 843–47. doi:10.1126/science.291.5505.843.

Mitrovich, QM, and P Anderson. 2000. 'Unproductively Spliced Ribosomal Protein MRNAs Are Natural Targets of MRNA Surveillance in C. Elegans.'. *Genes Dev* 14: 2173–84.

Miyoshi, H, JW Perfield, SC Souza, WJ Shen, HH Zhang, ZS Stancheva, FB Kraemer, MS Obin, AS Greenberg. 2007. 'Control of Adipose Triglyceride Lipase Action by Serine 517 of Perilipin A Globally Regulates Protein Kinase A-Stimulated Lipolysis in Adipocytes.'. *J Biol Chem* 282: 996–1002.

Miyoshi, H, SC Souza, HH Zhang, KJ Strissel, MA Christoffolete, J Kovsan, A Rudich, et al. 2006. 'Perilipin Promotes Hormone-Sensitive Lipase-Mediated Adipocyte Lipolysis via Phosphorylation-Dependent and -Independent Mechanisms.'. *J Biol Chem* 281: 15837–44.

Moghal, N, and PW Sternberg. 2003. 'A Component of the Transcriptional Mediator Complex Inhibits RAS-Dependent Vulval Fate Specification in C. Elegans.'. *Development* 130: 57–69.

Mulders, JW, W Hendriks, WM Blankesteijn, H Bloemendal, Jong WW de. 1988. 'Lambda-Crystallin, a Major Rabbit Lens Protein, Is Related to Hydroxyacyl-Coenzyme A Dehydrogenases.'. *J Biol Chem* 263: 15462–66.

Mullaney, BC, and K Ashrafi. 2009. 'C. Elegans Fat Storage and Metabolic Regulation.'. *Biochim Biophys Acta* 1791: 474–78.

Murphy, DJ. 1990. 'Storage Lipid Bodies in Plants and Other Organisms.'. *Prog Lipid Res* 29: 299–324.

Na, H, P Zhang, Y Chen, X Zhu, Y Liu, Y Liu, K Xie, et al. 2015. 'Identification of Lipid Droplet Structure-like/Resident Proteins in Caenorhabditis Elegans.'. *Biochim Biophys Acta* 1853: 2481–91.

Näär, AM., PA Beaurang, S Zhou, S Abraham, W Solomon, R Tjian. 1999. 'Composite Co-Activator ARC Mediates Chromatin-Directed Transcriptional Activation.'. *Nature* 398 (6730). Springer Nature: 828–32. doi:10.1038/19789.

Nagalingam, A, MTighiouart, L Ryden, L Joseph, G Landberg, NK Saxena, D Sharma. 2012. 'Med1 Plays a Critical Role in the Development of Tamoxifen Resistance.'. *Carcinogenesis* 33 (4). Oxford University Press (OUP): 918–30. doi:10.1093/carcin/bgs105.

Nagulapalli, M, S Maji, N Dwivedi, P Dahiya, JK Thakur. 2015. 'Evolution of Disorder in Mediator Complex and Its Functional Relevance.'. *Nucleic Acids Research* 44 (4). Oxford University Press (OUP): 1591–1612. doi:10.1093/nar/gkv1135.

Nagy, L, HY Kao, JD Love, C Li, E Banayo, JT Gooch, V Krishna, K Chatterjee, RM Evans, JWR Schwabe. 1999. 'Mechanism of Corepressor Binding and Release from Nuclear Hormone Receptors.'. *Genes & Development* 13 (24). Cold Spring Harbor Laboratory: 3209–16. doi:10.1101/gad.13.24.3209.

Nemet, J, B Jelicic, I Rubelj, M Sopta. 2014. 'The Two Faces of Cdk8 a Positive/Negative Regulator of Transcription.'. *Biochimie* 97 (February). Elsevier BV: 22–27. doi:10.1016/j.biochi.2013.10.004.

Nolte, RT, GB Wisely, S Westin, JE Cobb, MH Lambert, R Kurokawa, MG Rosenfeld, TM Willson, CK Glass, MV Milburn. 1998. 'Ligand Binding and Co-Activator Assembly of the Peroxisome Proliferator-Activated Receptor-Gamma.'. *Nature* 395: 137–43.

Notredame, C, DG Higgins, J Heringa. 2000. 'T-Coffee: A Novel Method for Fast and Accurate Multiple Sequence Alignment.'. *J Mol Biol* 302: 205–17.

Novotný, JP, AA Chughtai, M Kostrouchová, V Kostrouchová, D Kostrouch, F Kaššák, R Kaňa, B Schierwater, M Kostrouchová, Z Kostrouch. 2017. 'Trichoplax adhaerens reveals a network of nuclear receptors sensitive to 9-*cis*-retinoic acid at the base of metazoan evolution.'. PeerJ 5, e3789 (2017).

O'Rourke, EJ, AA Soukas, CE Carr, G Ruvkun. 2009. '*C. Elegans* Major Fats Are Stored in Vesicles Distinct from Lysosome-Related Organelles.'. *Cell Metab* 10: 430–35.

Ohsaki, Y, T Kawai, Y Yoshikawa, J Cheng, E Jokitalo, and T Fujimoto. 2016. 'PML Isoform II Plays a Critical Role in Nuclear Lipid Droplet Formation.'. *J Cell Biol* 212: 29–38.

Olsson, H, P Strålfors, P Belfrage. 1986. 'Phosphorylation of the Basal Site of Hormone-Sensitive Lipase by Glycogen Synthase Kinase-4.'. *FEBS Lett* 209: 175–80.

Olzmann, JA, and P Carvalho. 2018. 'Dynamics and Functions of Lipid Droplets.'. *Nat Rev Mol Cell Biol*.

Paix, A, A Folkmann, D Rasoloson, G Seydoux. 2015. 'High Efficiency, Homology-Directed Genome Editing in Caenorhabditis Elegans Using CRISPR-Cas9 Ribonucleoprotein Complexes.'. *Genetics* 201: 47–54.

Park, SW, G Li, YP Lin, MJ Barrero, K Ge, RG Roeder, LN Wei. 2005. 'Thyroid Hormone-Induced Juxtaposition of Regulatory Elements/Factors and Chromatin Remodeling of Crabp1 Dependent on MED1/TRAP220.'. *Molecular Cell* 19 (5). Elsevier BV: 643–53. doi:10.1016/j.molcel.2005.08.008.

Pei, J, and NV Grishin. 2007. 'PROMALS: towards Accurate Multiple Sequence Alignments of Distantly Related Proteins.'. *Bioinformatics* 23: 802–8.

Pei, J, BH Kim, M Tang, NV Grishin. 2007a. 'PROMALS Web Server for Accurate Multiple Protein Sequence Alignments.'. *Nucleic Acids Res* 35: W649–52.

Pei, J, BH Kim, NV Grishin. 2008. 'PROMALS3D: a Tool for Multiple Protein Sequence and Structure Alignments.'. *Nucleic Acids Res* 36: 2295–2300.

Perissi, V, A Aggarwal, CK Glass, DW Rose, MG Rosenfeld. 2004. 'A Corepressor/Coactivator Exchange Complex Required for Transcriptional Activation by Nuclear Receptors and Other Regulated Transcription Factors.'. *Cell* 116 (4). Elsevier BV: 511–26. doi:10.1016/s0092-8674(04)00133-3.

Petty, KJ, B Desvergne, T Mitsuhashi, VM Nikodem. 1990. Identification of a thyroid hormone response element in the malic enzyme gene.'. J Biol Chem 265, 7395-400.

Phelan, CA, RT Gampe, MH Lambert, DJ Parks, V Montana, J Bynum, TM Broderick, et al. 2010. 'Structure of Rev-erbalpha Bound to N-CoR Reveals a Unique Mechanism of Nuclear ReceptorCo-Repressor Interaction.'. *Nature Structural & Molecular Biology* 17 (7). Springer Nature: 808–14. doi:10.1038/nsmb.1860.

Phillips, T, L Hoopes. 2008. 'Transcription Factors and Transcriptional Control in Eukaryotic Cells.'. *Nature Education* 1(1):119.

Plaschka, C, L Larivière, L Wenzeck, M Seizl, M Hemann, D Tegunov, EV Petrotchenko, et al. 2015. 'Architecture of the RNA Polymerase IIMediator Core Initiation Complex.'. *Nature* 518 (7539). Springer Nature: 376–80. doi:10.1038/nature14229.

Pollak, NM, D Jaeger, S Kolleritsch, R Zimmermann, R Zechner, A Lass, G Haemmerle. 2015. 'The Interplay of Protein Kinase A and Perilipin 5 Regulates Cardiac Lipolysis.'. *J Biol Chem* 290: 1295–1306.

Poss, ZC, CC Ebmeier, DJ Taatjes. 2013. 'The Mediator Complex and Transcription Regulation.'. *Critical Reviews in Biochemistry and Molecular Biology* 48 (6). Informa UK Limited: 575–608. doi:10.3109/10409238.2013.840259.

Potcoava, MC, GL Futia, J Aughenbaugh, IR Schlaepfer, EA Gibson. 2014. 'Raman and Coherent Anti-Stokes Raman Scattering Microscopy Studies of Changes in Lipid Content and Composition in Hormone-Treated Breast and Prostate Cancer Cells.'. *J Biomed Opt* 19: 111605.

Praitis, V, E Casey, D Collar, J Austin. 2001. 'Creation of Low-Copy Integrated Transgenic Lines in Caenorhabditis Elegans.'. *Genetics* 157: 1217–26.

Pratt, WB. 1997. 'Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones.'. *Endocrine Reviews* 18 (3). The Endocrine Society: 306–60. doi:10.1210/er.18.3.306.

Privalsky, ML. 2004. 'The Role of Corepressors in Transcriptional Regulation by Nuclear Hormone Receptors.'. *Annual Review of Physiology* 66 (1). Annual Reviews: 315–60. doi:10.1146/annurev.physiol.66.032802.155556.

Pugh, BF, and R Tjian. 1991. 'Transcription from a TATA-Less Promoter Requires a Multisubunit TFIID Complex.'. *Genes & Development* 5 (11). Cold Spring Harbor Laboratory: 1935–45. doi:10.1101/gad.5.11.1935.

Punta, M, PC Coggill, RY Eberhardt, J Mistry, J Tate, C Boursnell, N Pang, et al. 2012. 'The Pfam Protein Families Database.'. *Nucleic Acids Res* 40: D290–301.

Rachez, C, BD Lemon, Z Suldan, V Bromleigh, M Gamble, AM Näär, H Erdjument-Bromage, P Tempst, LP Freedman. 1999. 'Ligand-Dependent Transcription Activation by Nuclear Receptors Requires the DRIP Complex.'. *Nature* 398 (6730). Springer Nature: 824–28. doi:10.1038/19783.

Rachez, C, Z Suldan, J Ward, CPB Chang, D Burakov, H Erdjument-Bromage, P Tempst, LP Freedman. 1998. 'A Novel Protein Complex That Interacts with the Vitamin D3 Receptor in a Ligand-Dependent Manner and Enhances VDR Transactivation in a Cell-Free System.'. *Genes & Development* 12 (12). Cold Spring Harbor Laboratory: 1787–1800. doi:10.1101/gad.12.12.1787.

Rastinejad, F, P Huang, V Chandra, and S Khorasanizadeh. 2013. 'Understanding Nuclear Receptor Form and Function Using Structural Biology.'. *J Mol Endocrinol* 51: T1–T21.

Rastinejad, F, V Ollendorff, I Polikarpov. 2015. 'Nuclear Receptor Full-Length Architectures: Confronting Myth and Illusion with High Resolution.'. *Trends in Biochemical Sciences* 40 (1). Elsevier BV: 16–24. doi:10.1016/j.tibs.2014.10.011.

Ratledge, C. 1991. 'Microorganisms for Lipids.'. *Acta Biotechnologica* 11 (5). Wiley: 429–38. doi:10.1002/abio.370110506.

Reitzel, AM, and AM Tarrant. 2009. 'Nuclear Receptor Complement of the Cnidarian Nematostella Vectensis: Phylogenetic Relationships and Developmental Expression Patterns.'. *BMC Evol Biol* 9: 230.

Reitzel, AM, J Macrander, D Mane-Padros, B Fang, FM Sladek, AM Tarrant. 2018. 'Conservation of DNA and ligand binding properties of retinoid X receptor from the placozoan *Trichoplax adhaerens* to human.'. J Steroid Biochem Mol Biol184, 3-10.

Remmert, M, A Biegert, A Hauser, J Söding. 2011. 'HHblits: Lightning-Fast Iterative Protein Sequence Searching by HMM-HMM Alignment.'. *Nat Methods* 9: 173–75.

Ringrose, JH, HW van den Toorn, M Eitel, H Post, P Neerincx, B Schierwater, AF Altelaar, AJ Heck. 2013. 'Deep proteome profiling of *Trichoplax adhaerens* reveals remarkable features at the origin of metazoan multicellularity.'. Nat Commun 4, 1408.

Robert, V, and JL Bessereau. 2007. 'Targeted Engineering of the Caenorhabditis Elegans Genome Following Mos1-Triggered Chromosomal Breaks.'. *EMBO J* 26: 170–83.

Robinson-Rechavi, M, CV Maina, CR Gissendanner, V Laudet, A Sluder. 2005. 'Explosive Lineage-Specific Expansion of the Orphan Nuclear Receptor HNF4 in Nematodes.'. *Journal of Molecular Evolution* 60 (5). Springer Nature: 577–86. doi:10.1007/s00239-004-0175-8.

Robinson-Rechavi, M. 2003. 'The Nuclear Receptor Superfamily.'. *Journal of Cell Science* 116 (4). The Company of Biologists: 585–86. doi:10.1242/jcs.00247.

Robinson, PJ, MJ Trnka, R Pellarin, CH Greenberg, DA Bushnell, R Davis, AL Burlingame, A Sali, RD Kornberg. 2015. 'Molecular Architecture of the Yeast Mediator Complex.'. *ELife* 4 (September). eLife Sciences Organisation Ltd. doi:10.7554/elife.08719.

Rowe, ER, ML Mimmack, AD Barbosa, A Haider, I Isaac, MM Ouberai, AR Thiam, et al. 2016. 'Conserved Amphipathic Helices Mediate Lipid Droplet Targeting of Perilipins 13.'. *Journal of Biological Chemistry* 291 (13). American Society for Biochemistry & Molecular Biology (ASBMB): 6664–78. doi:10.1074/jbc.m115.691048.

Ruthmann, A, G Behrendt, R Wahl. 1986. 'The ventral epithelium of Trichoplax adhaerens (Placozoa): Cytoskeletal structures cell contacts and endocytosis'.' Zoomorphology 106, 115–122 Springer Nature.

Rydel, TJ, JM Williams, E Krieger, F Moshiri, WC Stallings, SM Brown, JC Pershing, JP Purcell, MF Alibhai. 2003. 'The Crystal Structure, Mutagenesis, and Activity Studies Reveal That Patatin Is a Lipid Acyl Hydrolase with a Ser-Asp Catalytic Dyad.'. *Biochemistry* 42: 6696–6708.

Sahu, R, S Kaushik, CC Clement, ES Cannizzo, B Scharf, A Follenzi, I Potolicchio, E Nieves, AM Cuervo, L Santambrogio. 2011. 'Microautophagy of Cytosolic Proteins by Late Endosomes.'. *Dev Cell* 20: 131–39.

Santambrogio, L, and AM Cuervo. 2011. 'Chasing the Elusive Mammalian Microautophagy.'. *Autophagy* 7: 652–54.

Sap, J, A Muñoz, J Schmitt, H Stunnenberg, B Vennström. 1989. 'Repression of Transcription Mediated at a Thyroid Hormone Response Element by the v-Erb-A Oncogene Product.'. *Nature* 340 (6230). Springer Nature: 242–44. doi:10.1038/340242a0.

Sato, S, C Tomomori-Sato, TJ Parmely, L Florens, B Zybailov, SK Swanson, CA Banks, et al. 2004. 'A Set of Consensus Mammalian Mediator Subunits Identified by Multidimensional Protein Identification Technology.'. *Mol Cell* 14: 685–91.

Schierwater, B, D de Jong, R Desalle. 2009. 'Placozoa and the evolution of Metazoa and intrasomatic cell differentiation.'. Int J Biochem Cell Biol 41, 370-9.

Schierwater, B, M Eitel, W Jakob, HJ Osigus, H Hadrys, SL Dellaporta, SO Kolokotronis, R DeSalle. 2009a. 'Concatenated Analysis Sheds Light on Early Metazoan Evolution and Fuels a Modern Urmetazoon Hypothesis.'. PLoS Biology 7, e1000020 Public Library of Science (PLoS).

Schultz, J, F Milpetz, P Bork, CP Ponting. 1998. 'SMART a simple modular architecture research tool: Identification of signaling domains.'. Proceedings of the National Academy of Sciences 95, 5857–5864.

Schupp, M, and MA Lazar. 2010. 'Endogenous Ligands for Nuclear Receptors: Digging Deeper.'. *J Biol Chem* 285: 40409–15.

Schweiger, M, G Schoiswohl, A Lass, FP Radner, G Haemmerle, R Malli, W Graier, et al. 2008. 'The C-Terminal Region of Human Adipose Triglyceride Lipase Affects Enzyme Activity and Lipid Droplet Binding.'. *J Biol Chem* 283: 17211–20.

Segraves, WA. 1991. 'Something Old, Some Things New: the Steroid Receptor Superfamily in *Drosophila*.'. *Cell* 67: 225–28.

Sever, R, and CK Glass. 2013. 'Signaling by Nuclear Receptors.'. *Cold Spring Harbor Perspectives in Biology* 5 (3). Cold Spring Harbor Laboratory: a016709–a016709. doi:10.1101/cshperspect.a016709.

Shen, WJ, S Patel, H Miyoshi, AS Greenberg, FB Kraemer. 2009. 'Functional Interaction of Hormone-Sensitive Lipase and Perilipin in Lipolysis.'. *J Lipid Res* 50: 2306–13.

Shu, X, J Chan, RO Ryan, TM Forte. 2007. 'Apolipoprotein A-V Association with Intracellular Lipid Droplets.'. *Journal of Lipid Research* 48 (7). American Society for Biochemistry & Molecular Biology (ASBMB): 1445–50. doi:10.1194/jlr.c700002-jlr200.

Siegler, H, O Valerius, T Ischebeck, J Popko, NJ Tourasse, O Vallon, I Khozin-Goldberg, GH Braus, and I Feussner. 2017. 'Analysis of the Lipid Body Proteome of the Oleaginous Alga Lobosphaera Incisa.'. *BMC Plant Biol* 17: 98.

Sievers, F, A Wilm, D Dineen, TJ Gibson, K Karplus, W Li, R Lopez, H McWilliam, M Remmert, J Soding, JD Thompson, DG Higgins. 2014. 'Fast scalable generation of high-quality protein multiple sequence alignments using Clustal Omega.'. Molecular Systems Biology 7, 539–539 EMBO.

Signorovitch, AY, SL Dellaporta, LW Buss. 2005. 'Molecular signatures for sex in the Placozoa.'. Proceedings of the National Academy of Sciences 102, 15518–15522 Proceedings of the National Academy of Sciences**.**

Singh, R, and AM Cuervo. 2012. 'Lipophagy: Connecting Autophagy and Lipid Metabolism.'. *Int J Cell Biol* 2012: 282041.

Singh, R, S Kaushik, Y Wang, Y Xiang, I Novak, M Komatsu, K Tanaka, AM Cuervo, MJ Czaja. 2009. 'Autophagy Regulates Lipid Metabolism.'. *Nature* 458: 1131–35.

Smale, ST, and JT Kadonaga. 2003. 'The RNA Polymerase II Core Promoter.'. *Annual Review of Biochemistry* 72 (1). Annual Reviews: 449–79. doi:10.1146/annurev.biochem.72.121801.161520.

Smith, CL, F Varoqueaux, M Kittelmann, RN Azzam, B Cooper, CA Winters, M Eitel, D Fasshauer, TS Reese. 2014. 'Novel cell types, neurosecretory cells, and body plan of the early-diverging metazoan *Trichoplax adhaerens.*'. Curr Biol24, 1565-1572.

Söding, J, A Biegert, AN Lupas. 2005. 'The HHpred Interactive Server for Protein Homology Detection and Structure Prediction.'. *Nucleic Acids Res* 33: W244–8.

Soukas, AA, EA Kane, CE Carr, JA Melo, G Ruvkun. 2009. 'Rictor/TORC2 Regulates Fat Metabolism, Feeding, Growth, and Life Span in Caenorhabditis Elegans.'. *Genes Dev* 23: 496–511.

Soutourina, J, S Wydau, Y Ambroise, C Boschiero, M Werner. 2011. 'Direct Interaction of RNA Polymerase II and Mediator Required for Transcription in Vivo.'. *Science* 331 (6023). American Association for the Advancement of Science (AAAS): 1451–54. doi:10.1126/science.1200188.

Soutourina, J. 2017. 'Transcription Regulation by the Mediator Complex.'. *Nature Reviews Molecular Cell Biology* 19 (4). Springer Nature: 262–74. doi:10.1038/nrm.2017.115.

Spieth, J. 2006. 'Overview of Gene Structure.'. *WormBook.* doi:10.1895/wormbook.1.65.1.

Srinivasan, J, F Kaplan, R Ajredini, C Zachariah, HT Alborn, PE Teal, RU Malik, AS Edison, PW Sternberg, FC Schroeder. 2008. 'A Blend of Small Molecules Regulates Both Mating and Development in Caenorhabditis Elegans.'. *Nature* 454: 1115–18.

Srivastava, M, E Begovic, J Chapman, NH Putnam, U Hellsten, T Kawashima, A Kuo, et al. 2008. 'The Trichoplax Genome and the Nature of Placozoans.'. *Nature* 454: 955–60.

Stéphane, JF Dufayard, V Lefort, M Anisimova, W Hordijk, O Gascuel. 2010. 'New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0.'. Systematic Biology 59, 307–321 Oxford University Press (OUP).

Strahl, BD, and CD Allis. 2000. 'The Language of Covalent Histone Modifications.'. *Nature* 403: 41–45.

Su, CL, C Sztalryd, JA Contreras, C Holm, AR Kimmel, C Londos. 2003. 'Mutational Analysis of the Hormone-Sensitive Lipase Translocation Reaction in Adipocytes.'. *J Biol Chem* 278: 43615–19.

Sztalryd, C, and DL Brasaemle. 2017. 'The Perilipin Family of Lipid Droplet Proteins: Gatekeepers of Intracellular Lipolysis.'. *Biochim Biophys Acta* 1862: 1221–32.

Sztalryd, C, G Xu, H Dorward, JT Tansey, JA Contreras, AR Kimmel, and C Londos. 2003. 'Perilipin A Is Essential for the Translocation of Hormone-Sensitive Lipase during Lipolytic Activation.'. *J Cell Biol* 161: 1093–1103.

Taatjes, DJ. 2002. 'Structure Function, and Activator-Induced Conformations of the CRSP Coactivator.'. *Science* 295 (5557). American Association for the Advancement of Science (AAAS): 1058–62. doi:10.1126/science.1065249.

Taatjes, DJ. 2010. 'The Human Mediator Complex: a Versatile Genome-Wide Regulator of Transcription.'. *Trends in Biochemical Sciences* 35 (6). Elsevier BV: 315–22. doi:10.1016/j.tibs.2010.02.004.

Tabara, H, RJ Hill, CC Mello, JR Priess, Y Kohara. 1999. 'Pos-1 Encodes a Cytoplasmic Zinc-Finger Protein Essential for Germline Specification in C. Elegans.'. *Development* 126: 1–11.

Takagi, Y, and RD Kornberg. 2005. 'Mediator as a General Transcription Factor.'. *Journal of Biological Chemistry* 281 (1). American Society for Biochemistry & Molecular Biology (ASBMB): 80–89. doi:10.1074/jbc.m508253200.

Takahashi, H, TJ Parmely, S Sato, C Tomomori-Sato, CAS Banks, SE Kong, H Szutorisz, et al. 2011. 'Human Mediator Subunit MED26 Functions as a Docking Site for Transcription Elongation Factors.'. *Cell* 146 (1). Elsevier BV: 92–104. doi:10.1016/j.cell.2011.06.005.

Targett-Adams, P, D Chambers, S Gledhill, RG Hope, JF Coy, A Girod, J McLauchlan. 2003. 'Live Cell Analysis and Targeting of the Lipid Droplet-Binding Adipocyte Differentiation-Related Protein.'. *Journal of Biological Chemistry* 278 (18). American Society for Biochemistry & Molecular Biology (ASBMB): 15998–6007. doi:10.1074/jbc.m211289200.

Taubert, S, JD Ward, and KR Yamamoto. 2011. 'Nuclear Hormone Receptors in Nematodes: Evolution and Function.'. *Molecular and Cellular Endocrinology* 334 (1-2). Elsevier BV: 49–55. doi:10.1016/j.mce.2010.04.021.

Taubert, S, MR Van Gilst, M Hansen, KR Yamamoto. 2006. 'A Mediator Subunit, MDT-15, Integrates Regulation of Fatty Acid Metabolism by NHR-49-Dependent and -Independent Pathways in C. Elegans.'. *Genes Dev* 20: 1137–49.

Tauchi-Sato, K, S Ozeki, T Houjou, R Taguchi, T Fujimoto. 2002. 'The Surface of Lipid Droplets Is a Phospholipid Monolayer with a Unique Fatty Acid Composition.'. *J Biol Chem* 277: 44507–12.

Taylor, SS, R Ilouz, P Zhang, AP Kornev. 2012. 'Assembly of Allosteric Macromolecular Switches: Lessons from PKA.'. *Nat Rev Mol Cell Biol* 13: 646–58.

Thompson, CM, AJ Koleske, DM Chao, RA Young. 1993. 'A Multisubunit Complex Associated with the RNA Polymerase II CTD and TATA-Binding Protein in Yeast.'. *Cell* 73: 1361–75.

Thompson, CM, and RA Young. 1995. 'General Requirement for RNA Polymerase II Holoenzymes in Vivo.'. *Proceedings of the National Academy of Sciences* 92 (10). Proceedings of the National Academy of Sciences: 4587–90. doi:10.1073/pnas.92.10.4587.

Timmons, L, DL Court, A Fire. 2001. 'Ingestion of Bacterially Expressed DsRNAs Can Produce Specific and Potent Genetic Interference in Caenorhabditis Elegans.'. *Gene* 263: 103–12.

Tirinato, L, F Pagliari, T Limongi, M Marini, A Falqui, J Seco, P Candeloro, C Liberale, FE Di. 2017. 'An Overview of Lipid Droplets in Cancer and Cancer Stem Cells.'. *Stem Cells Int* 2017: 1656053.

Tóth-Petróczy, A, I Simon, C Oldfield, Y Takagi, K Dunker, V Uversky, M Fuxreiter. 2009. 'Malleable Machines in Transcription Regulation: the Mediator Complex.'. *Biophysical Journal* 96 (3). Elsevier BV: 58a. doi:10.1016/j.bpj.2008.12.198.

Tsai, KL, C Tomomori-Sato, S Sato, RC Conaway, JW Conaway, FJ Asturias. 2014. 'Subunit Architecture and Functional Modular Rearrangements of the Transcriptional Mediator Complex.'. *Cell* 157 (6). Elsevier BV: 1430–44. doi:10.1016/j.cell.2014.05.015.

Tutter, AV, MP Kowalski, GA Baltus, V Iourgenko, M Labow, E Li, S Kadam. 2008. 'Role for Med12 in Regulation OfNanogand Nanog Target Genes.'. *Journal of Biological Chemistry* 284 (6). American Society for Biochemistry & Molecular Biology (ASBMB): 3709–18. doi:10.1074/jbc.m805677200.

Ueno, M, WJ Shen, S Patel, AS Greenberg, S Azhar, and FB Kraemer. 2013. 'Fat-Specific Protein 27 Modulates Nuclear Factor of Activated T Cells 5 and the Cellular Response to Stress.'. *J Lipid Res* 54: 734–43.

Uzbekov, R, and P Roingeard. 2013. 'Nuclear Lipid Droplets Identified by Electron Microscopy of Serial Sections.'. *BMC Res Notes* 6: 386.

van der Lee, R, M Buljan, B Lang, RJ Weatheritt, GW Daughdrill, AK Dunker, M Fuxreiter, et al. 2014. 'Classification of Intrinsically Disordered Regions and Proteins.'. *Chemical Reviews* 114 (13). American Chemical Society (ACS): 6589–6631. doi:10.1021/cr400525m.

Van Gilst, MR, H Hadjivassiliou, A Jolly, KR Yamamoto. 2005. 'Nuclear Hormone Receptor NHR-49 Controls Fat Consumption and Fatty Acid Composition in C. Elegans.'. *PLoS Biol* 3: e53.

Vaquerizas, JM, SK Kummerfeld, SA Teichmann, NM Luscombe. 2009. 'A Census of Human Transcription Factors: Function Expression and Evolution.'. *Nature Reviews Genetics* 10 (4). Springer Nature: 252–63. doi:10.1038/nrg2538.

Viswanadha, S, and C Londos. 2008. 'Determination of Lipolysis in Isolated Primary Adipocytes.'. *Methods Mol Biol* 456: 299–306.

Vohanka, J, K Simecková, E Machalová, F Behenský, MW Krause, Z Kostrouch, M Kostrouchová. 2010. 'Diversification of Fasting Regulated Transcription in a Cluster of Duplicated Nuclear Hormone Receptors in C. Elegans.'. *Gene Expr Patterns* 10: 227–36.

Vrablik, TL, VA Petyuk, EM Larson, RD Smith, JL Watts. 2015. 'Lipidomic and Proteomic Analysis of Caenorhabditis Elegans Lipid Droplets and Identification of ACS-4 as a Lipid Droplet-Associated Protein.'. *Biochim Biophys Acta* 1851: 1337–45.

Wagner, M, G Zollner, M Trauner. 2011. 'Nuclear Receptors in Liver Disease.'. *Hepatology* 53 (3). Wiley-Blackwell: 1023–34. doi:10.1002/hep.24148.

Wallberg, AE, S Yamamura, S Malik, BM Spiegelman, RG Roeder. 2003. 'Coordination of p300-Mediated Chromatin Remodeling and TRAP/Mediator Function through Coactivator PGC-1alpha.'. *Mol Cell* 12: 1137–49.

Wältermann, M, and A Steinbüchel. 2005. 'Neutral Lipid Bodies in Prokaryotes: Recent Insights into Structure, Formation, and Relationship to Eukaryotic Lipid Depots.'. *J Bacteriol* 187: 3607–19.

Walther, TC, and RV Jr Farese. 2012. 'Lipid Droplets and Cellular Lipid Metabolism.'. *Annu Rev Biochem* 81: 687–714.

Wang, CW. 2016. 'Lipid Droplets, Lipophagy, and beyond.'. *Biochim Biophys Acta* 1861: 793–805.

Wang, G, MA Balamotis, JL Stevens, Y Yamaguchi, H Handa, AJ Berk. 2005. 'Mediator Requirement for Both Recruitment and Postrecruitment Steps in Transcription Initiation.'. *Molecular Cell* 17 (5). Elsevier BV: 683–94. doi:10.1016/j.molcel.2005.02.010.

Wang, H, L Hu, K Dalen, H Dorward, A Marcinkiewicz, D Russell, D Gong, et al. 2009. 'Activation of Hormone-Sensitive Lipase Requires Two Steps, Protein Phosphorylation and Binding to the PAT-1 Domain of Lipid Droplet Coat Proteins.'. *J Biol Chem* 284: 32116–25.

Wang, H, M Bell, U Sreenivasan, H Hu, J Liu, K Dalen, C Londos, et al. 2011. 'Unique Regulation of Adipose Triglyceride Lipase (ATGL) by Perilipin 5, a Lipid Droplet-Associated Protein.'. *J Biol Chem* 286: 15707–15.

Wang, H, RM La, LS Qi. 2016. 'CRISPR/Cas9 in Genome Editing and Beyond.'. *Annu Rev Biochem* 85: 227–64.

Wang, L, WJ Zuercher, TG Consler, MH Lambert, AB Miller, LA Orband-Miller, DD McKee, TM Willson, RT Nolte. 2006. 'X-Ray Crystal Structures of the Estrogen-Related Receptor-gamma Ligand Binding Domain in Three Functional States Reveal the Molecular Basis of Small Molecule Regulation.'. *Journal of Biological Chemistry* 281 (49). American Society for Biochemistry & Molecular Biology (ASBMB): 37773–81. doi:10.1074/jbc.m608410200.

Wang, MC, EJ O'Rourke, G Ruvkun. 2008. 'Fat Metabolism Links Germline Stem Cells and Longevity in C. Elegans.'. *Science* 322: 957–60.

Wang, X, F Wu, Q Xie, H Wang, Y Wang, Y Yue, O Gahura, et al. 2012. 'SKIP Is a Component of the Spliceosome Linking Alternative Splicing and the Circadian Clock in Arabidopsis.'. *Plant Cell* 24: 3278–95.

Wang, X, N Yang, E Uno, RG Roeder, S Guo. 2006a. 'A Subunit of the Mediator Complex Regulates Vertebrate Neuronal Development.'. *Proceedings of the National Academy of Sciences* 103 (46). Proceedings of the National Academy of Sciences: 17284–89. doi:10.1073/pnas.0605414103.

Wang, Y, Y Fu, L Gao, G Zhu, J Liang, C Gao, B Huang, et al. 2010. 'Xenopus Skip Modulates Wnt/Beta-Catenin Signaling and Functions in Neural Crest Induction.'. *J Biol Chem* 285: 10890–901.

Ward, JD. 2015. 'Rapid and Precise Engineering of the Caenorhabditis Elegans Genome with Lethal Mutation Co-Conversion and Inactivation of NHEJ Repair.'. *Genetics* 199: 363–77.

Warfield, L, LM Tuttle, D Pacheco, RE Klevit, S Hahn. 2014. 'A Sequence-Specific Transcription Activator Motif and Powerful Synthetic Variants That Bind Mediator Using a Fuzzy Protein Interface.'. *Proceedings of the National Academy of Sciences* 111 (34). Proceedings of the National Academy of Sciences: E3506–E3513. doi:10.1073/pnas.1412088111.

Wärnmark, A, E Treuter, APH Wright, JA Gustafsson. 2003. 'Activation Functions 1 and 2 of Nuclear Receptors: Molecular Strategies for Transcriptional Activation.'. *Molecular Endocrinology* 17 (10). The Endocrine Society: 1901–9. doi:10.1210/me.2002-0384.

Watson, PJ, L Fairall, GM Santos, JWR Schwabe. 2012. 'Structure of HDAC3 Bound to Corepressor and Inositol Tetraphosphate.'. Protein Data Bank Rutgers University. doi:10.2210/pdb4a69/pdb.

Watson, PJ, L Fairall, JWR Schwabe. 2012a. 'Nuclear Hormone Receptor Co-Repressors: Structure and Function.'. *Molecular and Cellular Endocrinology* 348 (2). Elsevier BV: 440–49. doi:10.1016/j.mce.2011.08.033.

Watts, JL, and J Browse. 2002. 'Genetic Dissection of Polyunsaturated Fatty Acid Synthesis in Caenorhabditis Elegans.'. *Proc Natl Acad Sci U S A* 99: 5854–59.

Weigel, NL. 1996. 'Steroid Hormone Receptors and Their Regulation by Phosphorylation.'. *Biochem J* 319 (Pt 3): 657–67.

Welte, MA, S Cermelli, J Griner, A Viera, Y Guo, DH Kim, JG Gindhart, SP Gross. 2005. 'Regulation of Lipid-Droplet Transport by the Perilipin Homolog LSD2.'. *Curr Biol* 15: 1266–75.

Wiederhold, T, MF Lee, M James, R Neujahr, N Smith, A Murthy, J Hartwig, JF Gusella, V Ramesh. 2004. 'Magicin, a Novel Cytoskeletal Protein Associates with the NF2 Tumor Suppressor Merlin and Grb2.'. *Oncogene* 23: 8815–25.

Wilfling, F, AR Thiam, MJ Olarte, J Wang, R Beck, TJ Gould, ES Allgeyer, et al. 2014. 'Arf1/COPI Machinery Acts Directly on Lipid Droplets and Enables Their Connection to the ER for Protein Targeting.'. *Elife* 3: e01607.

Wilfling, F, H Wang, JT Haas, N Krahmer, TJ Gould, A Uchida, JX Cheng, et al. 2013. 'Triacylglycerol Synthesis Enzymes Mediate Lipid Droplet Growth by Relocalizing from the ER to Lipid Droplets.'. *Dev Cell* 24: 384–99.

Wilson, PA, SD Gardner, NM Lambie, SA Commans, DJ Crowther. 2006. 'Characterization of the Human Patatin-like Phospholipase Family.'. *J Lipid Res* 47: 1940–49.

Wood, WB. 1988. 'Determination of Pattern and Fate in Early Embryos of Caenorhabditis Elegans.'. *Dev Biol (N Y 1985)* 5: 57–78.

Xie, M, and R Roy. 2015. 'The Causative Gene in Chanarian Dorfman Syndrome Regulates Lipid Droplet Homeostasis in C. Elegans.'. *PLoS Genet* 11: e1005284.

Xu, H Eric, TB Stanley, VG Montana, MH Lambert, BG Shearer, JE Cobb, DD McKee, et al. 2002. 'Structural Basis for Antagonist-Mediated Recruitment of Nuclear Co-Repressors by PPARalpha.'. *Nature* 415 (6873). Springer Nature: 813–17. doi:10.1038/415813a.

Yamaguchi, T, N Omatsu, S Matsushita, T Osumi. 2004. 'CGI-58 Interacts with Perilipin and is localized to Lipid Droplets. Possible Involvement of CGI-58 Mislocalization in Chanarin-Dorfman Syndrome.'. *J Biol Chem* 279: 30490–97.

Yang, F, BW Vought, JS Satterlee, AK Walker, ZY Jim Sun, JL Watts, R DeBeaumont, et al. 2006. 'An ARC/Mediator Subunit Required for SREBP Control of Cholesterol and Lipid Homeostasis.'. *Nature* 442 (7103). Springer Nature: 700–704. doi:10.1038/nature04942.

Yang, L, Y Ding, Y Chen, S Zhang, C Huo, Y Wang, J Yu, et al. 2012. 'The Proteomics of Lipid Droplets: Structure, Dynamics, and Functions of the Organelle Conserved from Bacteria to Humans.'. *J Lipid Res* 53: 1245–53.

Yang, X, X Lu, M Lombès, GB Rha, YI Chi, TM Guerin, EJ Smart, and J Liu. 2010. 'The G(0)/G(1) Switch Gene 2 Regulates Adipose Lipolysis through Association with Adipose Triglyceride Lipase.'. *Cell Metab* 11: 194–205.

Yang, Z, and DJ Klionsky. 2010. 'Eaten Alive: a History of Macroautophagy.'. *Nat Cell Biol* 12: 814–22.

Yoda, A, H Kouike, H Okano, H Sawa. 2005. 'Components of the Transcriptional Mediator Complex Are Required for Asymmetric Cell Division in C. Elegans.'. *Development* 132: 1885–93.

Zhang, C, DR Dowd, A Staal, C Gu, JB Lian, AJ van Wijnen, GS Stein, PN MacDonald. 2003. 'Nuclear Coactivator-62 KDa/Ski-Interacting Protein Is a Nuclear Matrix-Associated Coactivator That May Couple Vitamin D Receptor-Mediated Transcription and RNA Splicing.'. *J Biol Chem* 278: 35325–36.

Zhang, H, Y Wang, J Li, J Yu, J Pu, L Li, H Zhang, et al. 2011. 'Proteome of Skeletal Muscle Lipid Droplet Reveals Association with Mitochondria and Apolipoprotein a-I.'. *J Proteome Res* 10: 4757–68.

Zhang, P, H Na, Z Liu, S Zhang, P Xue, Y Chen, J Pu, et al. 2012. 'Proteomic Study and Marker Protein Identification of Caenorhabditis Elegans Lipid Droplets.'. *Mol Cell Proteomics* 11: 317–28.

Zhao, P, Z Zhang, H Ke, Y Yue, D Xue. 2014. 'Oligonucleotide-Based Targeted Gene Editing in C. Elegans via the CRISPR/Cas9 System.'. *Cell Res* 24: 247–50.

Zhou, S, M Fujimuro, JJ Hsieh, L Chen, A Miyamoto, G Weinmaster, and SD Hayward. 2000. 'SKIP, a CBF1-Associated Protein, Interacts with the Ankyrin Repeat Domain of NotchIC To Facilitate NotchIC Function.'. *Mol Cell Biol* 20: 2400–2410.

Zhu, Y, C Qi, S Jain, MS Rao, JK Reddy. 1997. 'Isolation and Characterization of PBP a Protein That Interacts with Peroxisome Proliferator-Activated Receptor.'. *Journal of Biological Chemistry* 272 (41). American Society for Biochemistry & Molecular Biology (ASBMB): 25500–25506. doi:10.1074/jbc.272.41.25500.

Zimmermann, R, JG Strauss, G Haemmerle, G Schoiswohl, R Birner-Gruenberger, M Riederer, A Lass, et al. 2004. 'Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride Lipase.'. *Science* 306: 1383–86.

Zumbusch, A, W Langbein, and P Borri. 2013. 'Nonlinear Vibrational Microscopy Applied to Lipid Biology.'. *Prog Lipid Res* 52: 615–32.