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# Gene expression regulation by nuclear receptors in a specific metabolic context – evolutionary perspective

# Regulace genové exprese jadernými receptory ve specifickém metabolickém kontextu – evoluční perspektiva

Disertační práce

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# Abstract

In animals, some of the most critical regulators of gene expression are nuclear hormone receptors (NRs) and their coregulators, specifically the Mediator complex. Of particular interest are the NRs implicated in metabolic and developmental regulation and in carcinogenesis: thyroid hormone receptors (TRs) and retinoid X receptors (RXRs). In this work, I venture to elucidate some aspects of gene expression regulation by these NRs: the degree of evolutionary conservation of their signalling pathways; the mechanisms of negative regulation by NRs; and possible implications of these findings for clinical medicine. State-of-the-art bioinformatical, genome editing and microscopic techniques are applied at three levels of animal evolution to study NRs and Mediator. Reverse genomics in human patients suffering from the syndrome of resistance to thyroid hormones type β are used to infer the structure and function of TRβ subdomains. Alignments, binding studies and *in vivo* experiments in *Trichoplax adhaerens* allowed identification of a close orthologue of human RXR at the basis of metazoan evolution. Employing database queries, genome editing and microscopy, we describe a correct orthologue of the Mediator subunit 28 in *Caenorhabditis elegans*, indicating a complete homology of the Mediator complex between nematodes and human. Analysing the results between species, we provide further indications that regulation by the NR-Mediator axis is conserved throughout *metazoans* and we propose a hypothetical working model of the negative regulation by NRs.



# Keywords

*Caenorhabditis elegans*, gene expression regulation, Mediator subunit 28 (MED28), negative regulation by TRs, nuclear receptors, retinoid X receptors (RXRs), syndrome of resistance to thyroid hormones – type β (RTHβ), thyroid hormone receptors (TRs), *Trichoplax adhaerens*.

# Abstrakt

Mezi nejdůležitější regulátory genové exprese u zvířat patří jaderné receptory (NRs) a jejich koregulátory, zejména Mediátorový komplex. Zvláštní zájem vzbuzují NRs zúčastňující se na metabolické a vývojové regulaci a na karcinogenesi: receptory hormonů štítné žlázy (TRs) a retinoidové X receptory (RXRs). Ve své práci se podjímám úkolu objasnit některé aspekty regulace genové exprese těmito NRs: míru evoluční konzervace jejich signalizačních drah; mechanismy negativní regulace jadernými receptory; a možné aplikace těchto objevů v klinické medicíně. Použil jsem bioinformatické a mikroskopické metody, včetně metod genové editace, a to na třech úrovních evoluce zvířat ke studiu vtahu NRs a Mediátoru. Reversní genomická analýza u lidských pacientů trpících syndromem rezistence k thyroidním hormonům je využita k posouzení struktury a funkce subdomén TRβ. Porovnání sekvencí, vázné studie a *in vivo* experimenty u *Trichoplax adhaerens* vedly k identifikaci blízkého ortologu lidského RXR na počátku evoluce zvířat. Použitím analýzy databází, editace genomu a mikroskopie jsme identifikovali skutečný ortolog mediátorové podjednotky 28 u *Caenorhabditis elegans*, což poukazuje na zásadní homologii Mediátorového komplexu mezi nematody a člověkem. Analýza vztahu mezi druhy posiluje koncept konzervace regulační osy NR-Mediator u všech *metazoa.* Na tomto základě navrhuji hypotetický funkční model negativní regulace NRs a diskutuji možnou roli TR, RXR a MED s dopady na pochopení karcinogenese a možné aplikace v molekulární a klinické onkologii.

# Klíčové pojmy

*Caenorhabditis elegans*, jaderné receptory (NRs), mediatorová podjednotka 28 (MED28), negativní regulace prostřednictvím TRs, regulace genové exprese, retinoidní X receptory (RXRs), syndrom resistence k thyroidním hormonum β (RTHβ), thyroidní receptory (TRs), *Trichoplax adhaerens*.

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# 1. Introduction

## 1.1. Equilibrium and integration of signalling

Cell is the most basic self-sustaining unit of life on Earth. Unicellular organisms developed mechanisms enabling them to sense and respond to changes in environment, such as presence or scarcity of food, sense of time and space, damage or danger. One common feature of these regulations in unicellular organisms is their implementation on an individual cellular level, *e.g.,* a sudden availability of food is sensed individually by each cell.

However, favourable cooperation between cells eventually brought about multicellularity in several complex, not completely understood processes (Grosberg & Strathmann, 2007). A prerequisite for multicellularity is intercellular communication. If multiple cells are to thrive together, their internal processes need not only respond to the needs of an individual cell but also to the needs of the whole multicellular organism. Intercellular signalling allows functional (and structural) specialisation of cells and allows the organism to react to external stimuli, as a whole.

Intercellular communication is based on an intricate network of signals. Where we once thought of intercellular signalling as of straight-line pathways, we now realise that all regulatory mechanisms are integrated. This integration is based on a vast network of logical circuits, mathematically not unlike the logical operations of complex computers (Rosenfeld et al., 2006). Even the most basic multicellular organisms are likely to possess the capacity to logically integrate signals and make basic decisions, not just reactions.

The model of signal integration is based on the possibility to alter a reaction to one input by the presence or absence of a second input. This form of a logical gate acts as the most basic unit of computational logic and, as it seems, of biological regulatory networks (Rosenfeld et al., 2006). The very basic model of such functioning must take into account the registering, processing, integration and response to internal and external signals.

Environmental signals might have various forms, from chemical gradients to mechanical stimuli to physical factors. Intercellular signals transmitting messages about homeostasis, cellular differentiation programs, metabolism, reproduction *etc*., take mostly the form of chemical gradients or electrochemical potentials. All these signals have thus different qualities and must first be translated into quantitatively measurable scalars of one or few variables.

First, cytoplasmic integration of incoming signals occurs. Signal is translated and unified thanks to a network of second messengers such as cyclic adenosine monophosphate (cAMP), $Ca^{2+}$ ions and protein phosphorylation. These partially account for direct cytoplasmic effects, partially proceed to the nucleus. Here, an ultimate level of integration takes place, where multiple pathways converge and interact with the pre-existing status of nucleoplasm. DNA sequences, nuclear proteins, their respective interactions, their modifications, as well as higher-level chromatin structure, all modulate the integration of incoming signals. As a result, expression of target genes is stimulated or suppressed, resulting in direct effects in the cell (*e.g.* cell differentiation) and in the formation of down-stream signals to other cells.

Of particular interest is the gene expression regulation on transcriptional level. It has been in the spotlight of biological research in the late 20th and early 21st century and up to this day, a considerable part of life sciences continues to try to elucidate the exact underlying mechanisms. The consensus model is based on individual interactions of sensors, integrators, chromatin structure and transcriptional machinery (Figure 1).

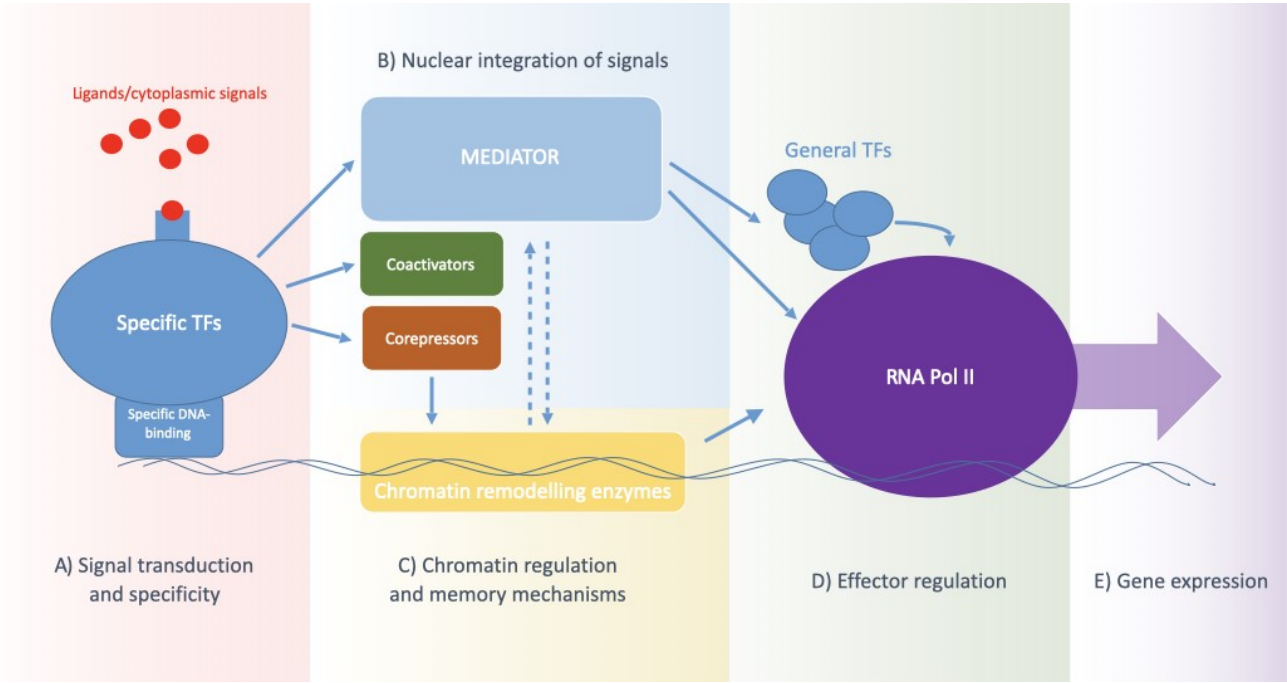


*Figure 1. Steps of signal processing and gene expression regulation in the nucleus. Created based on data from (Rosenfeld et al., 2006).*

Intercellular and environmental signals pass directly or indirectly via second messengers to the cell nucleus, where they are registered by a system of sensors called specific transcription factors (sTFs) (Figure 1A). Thereafter, sTFs transduce the signals to their coregulators. At this level, signals are

integrated, summed with information from other signalling pathways, as well as modified by the pre-existing state of chromatin (Figure 1B). The resulting decision issued from the signal integration is transmitted to the transcriptional machinery (Figure 1D) and may influence the state of chromatin, modifying subsequent integrations. This forms a sort of cellular memory (Figure 1C).

I will now discuss more specifically the individual players in these integrative pathways, the basic units responsible for modulating every single incoming signal, with a special attention paid to the scope of this thesis, the integration performed by the nuclear receptors (NRs) and their coregulators, especially the RNA polymerase II transcription mediator (the Mediator).

## 1.2. Specific transcription factors (sTFs)

Dispersed or DNA-bound sTFs have two crucial functions in transcriptional regulation: (1) sensing specific signals; and (2) addressing the signal to corresponding genomic sites, where they can be further transformed into alterations in specific gene expression (Latchman, 1997).

Protein-DNA interactions in the DNA-binding domains of sTFs direct these sTFs to individual binding sites dispersed throughout genomes, from where they can influence gene expression in proximate genes (P. J. Mitchell & Tjian, 1989). While we speak of proximity, one must always bear in mind that this proximity is not always unidimensional, *i.e.*, it is not always genes immediately upstream or downstream of the sTF binding site that are under the sphere of influence of the given sTF. Rather, chromatin is organized in a three-dimensional way, where loops of DNA from far reaches of one chromosome and even from various chromosomes come together in dynamic complexes bound together by proteins and RNAs and can be regulated by a given sTF (Ptashne & Gann, 1997).

Despite this complexity, sTFs and their modes of action are surprisingly evolutionarily conserved. It can be presumed that those sTFs which were employed first in the evolution are likely to regulate genes directly downstream in the nucleotide sequence of the chromosome, because more complex interactions of chromatin developed more recently in the evolution. Among these are nuclear hormone receptors (NRs).

### 1.2.1. Nuclear hormone receptors (NRs)

NRs are in the very centre of nuclear signal transduction and are some of the evolutionarily most ancient signalling proteins. Their remarkable compact structure endows them with great versatility. NRs are responsible for (1) specific DNA-binding at the site of regulated genes, (2) differential binding of co-activators and co-repressors, to directly modulate gene expression; and (3) all that depending on the concentration of ligands. NRs represent the absolute minimum needed to coordinate multicellular life in the most basic *metazoa* imaginable, while being indispensable in organisms as complicated as mammals (Evans, 1988).

Whilst much divergence has taken place during the 500 million years of animal multicellular evolution, NRs remain surprisingly homologous. It seems that they are so crucial for multicellular life, that all presently sequenced metazoan genomes contain NR genes and that in widely varied numbers. While a basic porifera species *Amphimedon queenslandica* has mere two identified NR genes, *Drosophila melanogaster* genome contains 21, human cells 46 and in the nematode *Caenorhabditis elegans* genome, a remarkable gene expansion has led to an astounding number of 270 NR genes (Figure 2) (Sladek, 2011). However, not all NRs and NR-like proteins act as conventional hormone receptors.

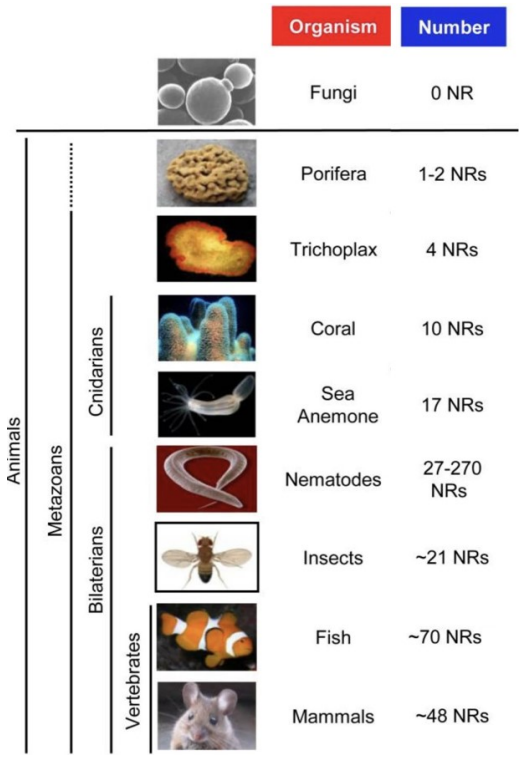


*Figure 2. Presence and number of nuclear receptors in representative phyla throughout eukaryotic life. Adapted with permission from (Sladek, 2011).*

Historically, the discovery and cloning of the first individual NRs in human has given rise to a field of molecular endocrinology (S. M. Hollenberg et al., 1985; Sherwood et al., 1971). The next twenty years of research identified dozens of proteins closely homologous to the known steroid hormone receptors. Surprisingly, for most of these, no endogenous ligands had initially been known and they were termed “orphan NRs”. Subsequent reverse endocrinology research, exploiting most notedly co-transfection essays, has identified endogenous ligands for many of these orphan NRs. After having identified endogenous ligands of previously orphan NRs, it became clear that most of these ligands are not conventional hormones but rather metabolites such as fatty acids, bile acids, oxysterols, *etc.* (Evans & Mangelsdorf, 2014).

Nevertheless, structural studies suggest that not all orphan NRs have the capacity to conditionally bind ligands and are likely to retain their orphan status. This is truer the deeper one retraces the evolution. The first NR is thought to have been formed via a gene fusion of pre-existing modules and might have been an orphan receptor (Barnett et al., 2000; Laudet, 1997), although this remains a subject of a debate.

### 1.2.2. Structure and classification of NRs

One of the most striking attributes of the NRs, is the degree of their structural conservation. Their shared structural organisation, *i.e.* their secondary and tertiary structure surpasses in homology their amino acid sequence conservation (Kumar & Thompson, 1999). This common structure includes presence of five basic domains: (A/B) Variable N-terminal domain (NTD) with or without activation function 1 (AF-1) module; (C) conserved DNA-binding domain (DBD) with $Zn^{2+}$-binding-residues of DNA-interacting P-box and a dimerizing D-box; (D) variable and little studied hinge region; (E) structurally conserved ligand-binding domain (LBD) consisting of 12 α-helices and 2-4 β-sheets, responsible not only for ligand binding, but also for dimerization, co-activator and co-repressor binding and for direct transactivation via its helix-12 AF2 module; and in some receptors a (F) variable C-terminal domain (Figure 3).

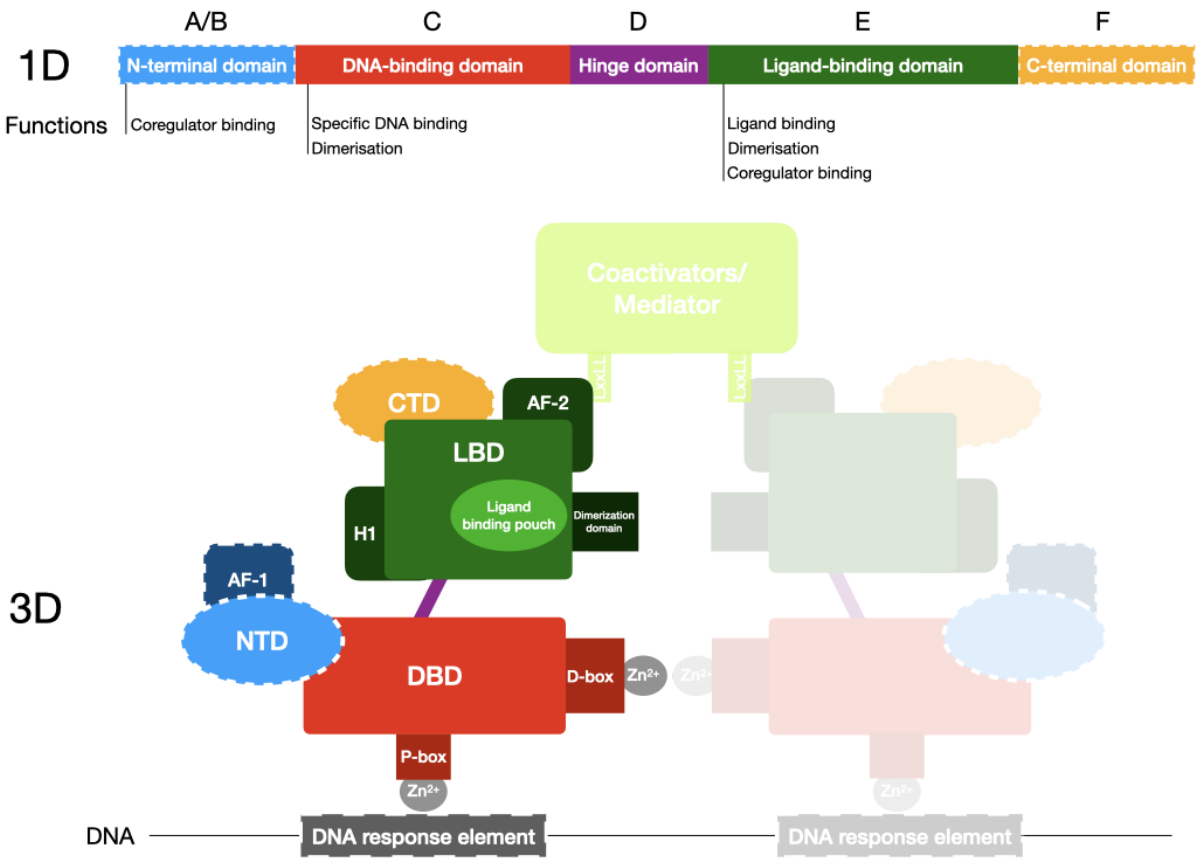


*Figure 3. Basic structure of nuclear receptors in the one-dimensional peptide sequence (1D) and in a three-dimensional structural model (3D) along with principal functions of individual domains. Figure modified from (Porter et al., 2019) originally published under the Creative Commons Attribution Licence.*

Based on the degree of evolutionary conservation and divergence, a classification of NR superfamily into seven subfamilies has been introduced (Auwerx et al., 1999). Whilst this classification takes primarily into account the conservation of their amino acid sequence, it also has far-reaching structural and functional consequences, as illustrated bellow. Subfamily 1 contains thyroid hormone receptor-related proteins of which notably: the thyroid hormone receptor itself (TR), all-*trans* retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and farnesoid X receptor (FXR), vitamin D receptor (VDR) and VDR-like receptors (PXR and CAR), as well as some other nematode and insect receptors (E78C-like and CNR14-like). Subfamily 2 contains the RXR and related hepatocyte nuclear factor 4 (HNF4), as well as chicken ovalbumin upstream promoter-transcription factor (COUP-TF). Subfamily 3 represents the classical steroid receptors such as the oestrogen receptor (ER) and 3-ketosteroid receptors: androgen receptor (AR), progesterone receptor (PR), mineralocorticoid and glucococorticoid receptor (MR and GR). Subfamilies 4 through

8 contain less well-studied NRs often with less homology and sometimes with separate mode of action (Table 1).

| NR subfamily | Particularities | Examples in human | Examples in *C. elegans* | Examples in *D. melanogaster* |
|---|---|---|---|---|
| **Subfamily 1** Thyroid Receptor-like | Homodimerization and heterodimerization with Subfamily 2 NRs on DR3, DR4 or DR5 elements. | Thyroid Receptors, Peroxisome proliferator-activated receptors (PPAR), Vitamin D Receptor (VDR) | SEX-1, DAF-12 (VDR homologue), NHR-23 (ROR homologue) | Ecdysone Receptor, DHR96 (VDR homologue), |
| **Subfamily 2** RXR-like | Heterodimerization as described above and homodimerization on DR1 elements. | Retinoid X Receptors (RXR), Hepatocyte Nuclear Factor 4 (HNF4), COUP-TF, TLX, PNR | NHR-49 (HNF4 homologue), NHR-67 (TLX homologue), UNC-55 (COUP homologue), FAX-1 (PNR homologue) | dmHNF4, Tailless (TLX homologue), Seven-up (COUP homologue), dmFAX-1 (PNR homologue) |
| **Subfamily 3** Steroid Receptors | Homodimerization on PD3 elements. Dominant role of NTD's AF1 module over AF2. Regulation by trafficking between cytoplasm and nucleus. | Estrogen Receptors, Glucocorticoid Receptor, Mineralocorticoid Receptor, Androgen Receptor | - | CG7404 |
| **Subfamily 4** | Monomer binding with C-terminal extension (CTE) on two elements. Dominant AF1 regardless of ligand binding in some NR4s. | Nerve Growth Factor IB, Nurr-1 | NHR-6 | DHR78 |
| **Subfamily 5** | Monomer binding with C-terminal extension (CTE) on two elements. | Steroidogenic factor 1 | NHR-25 | fushi tarazu factor I (FTZ-F1) |
| **Subfamily 6** | Homodimerization. | Germ cell nuclear factor | NHR-91 | DHR4 |
| **Subfamily 7** | Two tandem DNA-binding domains. Only present in flatworms, molluscs and some arthropods. | - | - | - |
| **Subfamily 8** | Only found in *Crassostrea gigas*. | - | - | - |

*Table 1. Nuclear Receptor classification into 8 subfamilies with most important examples. Modified with permission from (Auwerx et al., 1999) with data from (W. Huang et al., 2015; Kaur et al., 2015).*

The regulation by NRs is manifold and involves direct and indirect mechanisms. Essentially, in ligand-gated transcription factors, in order to regulate target gene expression, a given NR must (1) recognise and locally bind to its target genes; and (2) exercise its activating or suppressing effect.

Recruitment to individual genes is in large catered for by specific nucleotide sequences, or DNA response elements. However, the exact DNA element (six nucleotides) is same or similar for most NRs. Specificity is encoded solely by the linear organisation of two of these elements (Mangelsdorf

et al., 1995). Gene expression regulation by typical NRs canonically requires their homo- or hereto-dimerization. DNA response element sites in promoters of regulated genes typically coordinate this dimerization and thus direct appropriate NRs to their respective genes (Amoutzias et al., 2007; Pawlak et al., 2012). More specifically, steroid hormone receptors of subfamily 3 (ER, AR, PR, MR and GR) bind as homodimers to two palindromic half-sites spaced by three nucleotides. NRs of the subfamily 1 (TR, RAR, VDR, FXR, PXR, CAR, PPAR and LXR) bind mostly as heterodimers with RXR on two tandem (direct) repeat half-sites separated by three (VDR), four (TR) or five (RAR) random nucleotides. These are called direct repeat DR3, DR4 and DR5 sites. RXR and other subfamily 2 receptors can also bind as homodimers on two tandem repeat sites with a single spacer nucleotide (DR1). "Non-canonical" receptors of subfamily 4 and 5 (*e.g.,* liver receptor homologue LRH) contain the same single DBD but a small C-terminal extension (CTE) binds another element in neighbouring upstream major groove (McEwan, 2009). Even more interestingly, subfamily 7 NRs only present in flatworms, molluscs and some arthropods contain two tandem DBDs that bind response elements in two neighbouring major grooves (Wu, Niles, Hirai, et al., 2007).

As implied above, in dimerizing NRs, two parts of the NR molecule are responsible for the homo- or hetero-dimerization: (1) the Zinc-coordinated five amino acids of DBD D-box; and (2) a partially canonical part of the LBD. In the hetero-dimerising subfamily 1, these are clusters of polar-around-hydrophobic amino acids residues mapped to helices 9 and 10, occasionally with the assistance of helices 7 and 8 and loops connecting them. Subfamily 3 members homodimerize similarly, but helices 11 and 12 may also contribute. This specific dimerization in subfamilies 1 through 4 is crucial for their function (Amoutzias et al., 2007). Therefore, it makes sense that ligand binding contributes to this selective dimerization.

Depending on their ligand binding, NRs act as both *cis*- and *trans*-transcription factors (McEwan, 2009). Structurally speaking, also here two distinct domains of the NR molecule are important: (1) the variable AF1 module of the N-terminal domain in some receptors; and (2) distinct parts of the LBD, most importantly the AF2 subdomain.

The well-studied and structurally conserved AF2 subdomain is crucial for most transcriptional activation by NRs. Conventionally, ligand binding by an NR leads to the activation of AF2 by a rearrangement of the helix 12 and a formation of a hydrophobic groove consisting of helices 3, 4 and 5. Thus activated AF2 binds a consensual amino acid motif of three leucine residues separated by two random amino acids after the first leucine (LxxLL) by utilising hydrophobic bonds between the three leucines and the hydrophobic groove. Fidelity and stability of this bond is assured by a hydrophilic

clamp of a helix 12 glutamic acid residue and helix 3 lysine residue (McInerney et al., 1998). The LxxLL motif is the prevalent definition sequence of nuclear receptor coactivator proteins.

Contrarily, AF1 is surprisingly evolutionary diverse and no conserved molecular mechanics could have been deduced. N-terminal domains are generally intrinsically disordered but tend to form α-helices. DNA-binding by the DBD seems to cause molecular allosteric changes that reverberate into NTD structure and possibly thus exercise the AF1 activation (Simons et al., 2014).

The relative importance of AF1 and AF2 in transcriptional activation has likely shifted in the evolution. The length of N-terminal (A/B) domains in various NRs correlates with AF1 function (He et al., 2004). While most subfamily 1 and 2 members have short AF1 and their activation is mostly mediated by the AF2 subdomain, steroid receptors have long NTDs with pronounced AF1-activity. This suggests that in the subfamily 3 evolutionary branch, AF1 has taken over an important part of specific gene expression activation. Indeed, steroid receptors are regulated mostly by shuttling of the receptor between nucleus and cytoplasm via ligand-dependent binding of auxiliary heat-shock proteins (HSPs). Their AF2 subdomain might be, therefore, an evolutionary relic. Some other NRs such as the subfamily 4 Nurr-1 protein has strongly regulating AF1 that does not seem to be closely regulated by ligand binding (Nordzell et al., 2004). However, in most other families, ligand-dependent AF2 function is likely dominant.

Outside the DBDs and LBDs, little is known about the NR molecules. The hinge domain seems to play mostly structural role, but in some cases, important functions have also been localised here. In steroid receptors, it is the region of HSP binding. In heterodimerizing NRs (subfamilies 1 and 4), part of the hinge domain with the N-terminal part of LBD (helix 1), has been associated with corepressor binding (Apriletti et al., 1998). The C-terminal domain (CTD) is highly variable and its function is largely unknown. Many NRs do not have a CTD *per se* as a separate entity adjacent to the C-terminus of the conserved LBD. Notable exceptions are ER and HNF4α, both with large CTDs implicated in gene transcription regulation (Fonseca et al., 2020).

## 1.3. Signal integration and transcription – coregulators and Pol II

In the most eukaryotic organisms, regulation of transcription plays a dominant role in the regulation of gene expression. All protein-coding genes are transcribed by the DNA-dependent RNA-Polymerase II (Pol II), allowing for an amazing versatility in its regulation, and for an ultimate level of signal integration.

The crucial step in this regulation is transcription initiation. While there might be multiple mechanisms, the canonical model presumes the presence of a thymine/adenine-rich sequence in the upstream proximity of the first nucleotide to be transcribed, so called TATA-box. The Pol II holoenzyme cannot efficiently initiate transcription on its own. Rather several conserved assisting proteins and complexes collectively named Pol II general transcription factors (TFIIs) are required. Among these are TATA-binding protein (TBP) and TBP-associated factors ($TAF_{II}$-s).

Historically, NRs have been first shown to interact on their own with $TAF_{II}$-s and thus directly regulate transcription initiation (Furukawa & Tanese, 2000; Jacq et al., 1994). However, further studies have shown that this interaction was of minor importance and that supplementary proteins and complexes were necessary for efficient transcriptional regulation. Most of the firstly co-purified coregulators were coactivators relying on the internal histone-acetyltransferase (HAT) activity (see below), or interacting proteins binding to both, HAT complexes and NRs (Figure 4).

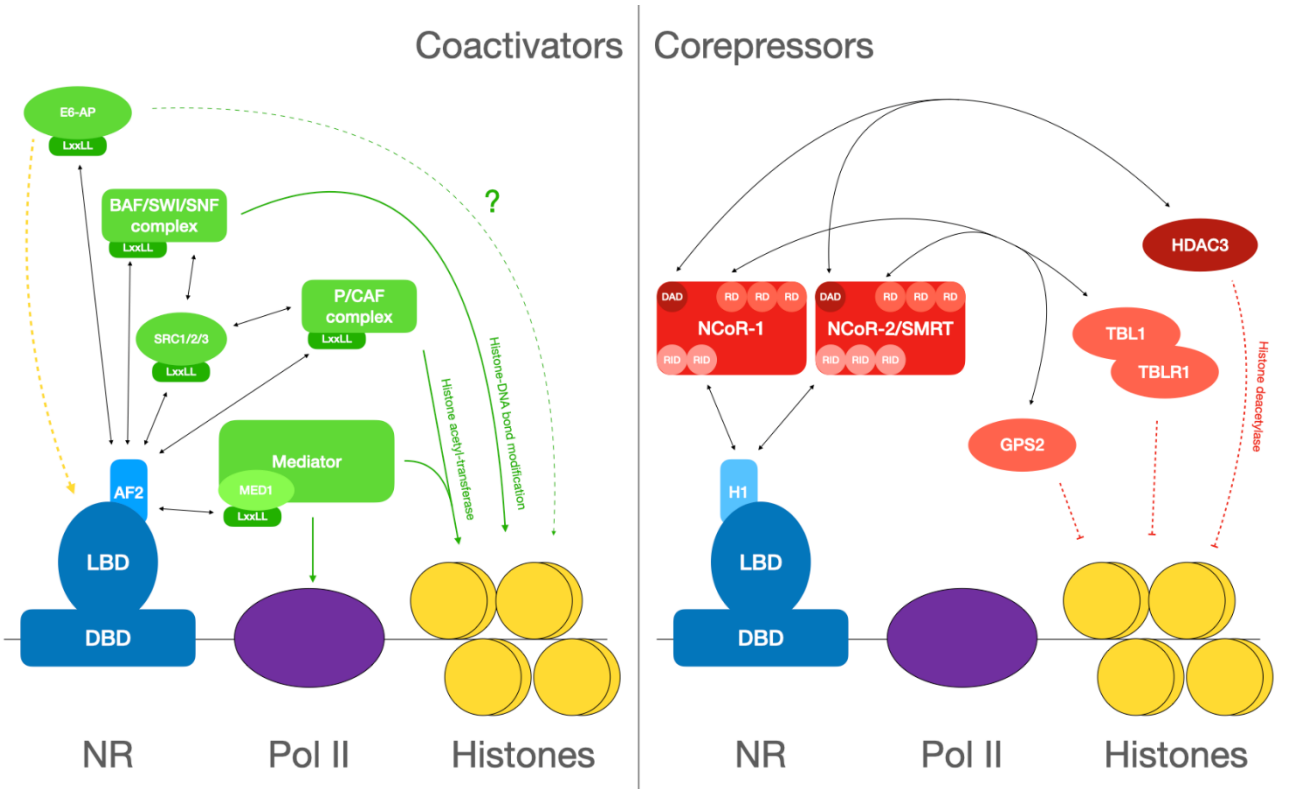


*Figure 4. Overview of the most important NR coactivators (A) and corepressors (B) and their canonical mechanical interactions. NR – nuclear receptor, Pol II – DNA Polymerase II, DBD – DNA-binding domain, LBD – ligand-binding domain, AF2 – activated function 2 subdomain, H1 – LBD helix 1, LxxLL – coactivator binding motif, RID – receptor-interacting domain, DAD – deacetylase-binding domain, RD – repression domain.*

The early work in *Saccharomyces cerevisiae* undertaken in the Roger David Kornberg lab suggested a presence of yet another mediator protein (Flanagan et al., 1991; Kelleher et al., 1990). Shortly thereafter, a large complex mediating TR-dependent (Fondell et al., 1996) and VDR-dependent (Rachez et al., 1998) transcription was described. In contrast to the previously discovered coactivators, it did not seem to possess direct histone acetyl-transferase activity. Individual protein subunits had been named after the NR it bound when discovered, with most subunits named TR associated proteins (TRAPs) and vitamin D receptor interacting proteins (DRIPs). The complex was originally named the DRIP/TRAP complex. While gradually uncovering more and more subunits of this immense complex, it became ever clearer that its activity is extremely important in regulation of transcription, that it is at the crossroads of nuclear signal integration and even more strikingly, that this complex is actually the missing direct link between sTFs (including NRs) and Pol II. Its name was eventually changed to the RNA Pol II transcription mediator complex, Mediator for short (Bourbon et al., 2004). The importance of these discoveries was highlighted by Kornberg being awarded the Nobel Prize in Chemistry in 2006 “for his studies of the molecular basis of eukaryotic transcription” (Nobel Committee for Chemistry, 2006).

### 1.3.1. The RNA Polymerase II transcription mediator complex (Mediator)

Mediator complex is an exceptionally intricate, yet remarkably evolutionarily conserved protein complex acting at the very centre of external signal integration and transmission. In the cybernetic model of intercellular communication, Mediator can be viewed as the central processing unit (CPU) with the main decisional power at the level of individual genes. While its name often makes readers confuse Mediator for an abstract concept of a coregulator, it is in fact a very well defined protein complex, necessary for virtually all eukaryotic transcription.

Mediator subunit genes are some of the most universal proteins of eukaryotic life with homologs present in all eukaryotic kingdoms: *protista* (Bourbon, 2008), animals including human (Soutourina, 2018), plants (Dolan & Chapple, 2017) and fungi (Moran et al., 2019). While these proteins share little homology in their nucleotide and amino acid sequences, their secondary, tertiary and quaternary structure is very well conserved with a shared complex structure and function between yeast and human and beyond (Figure 5).

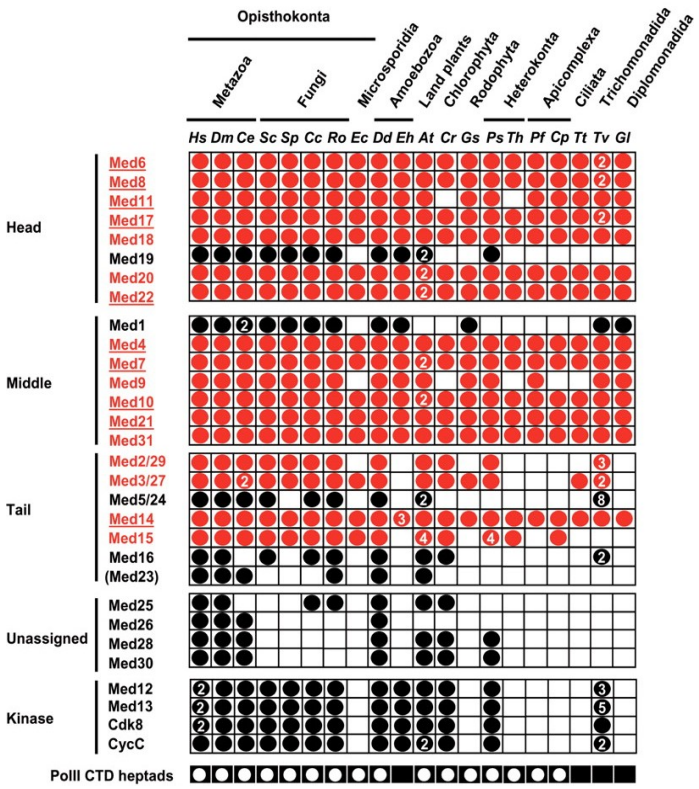


*Figure 5. Mediator subunit conservation throughout eukaryotic evolution. Individual taxons represented by model species noted in the top of the table, specifically: H. sapiens (Hs), D. melanogaster (Dm), C. elegans (Ce), S. cerevisiae (Sc), S. pombe (Sp), C. cinereus (Cc), R. oryzae (Ro), E. cuniculi (Ec), D. discoideum (Dd), E. histolytica (Eh), A. thaliana (At), C. reinhardtii (Cr), G. sulphuraria (Gs), P. sojae (Ps), T. pseudonana (Th), P. falciparum (Pf), C. parvum (Cp), T. thermophila (Tt), T. vaginalis (Tv) and G. lamblia (Gl). In red are marked subunits necessary for viability in yeast. Adapted with permission from (Bourbon, 2008).*

Based on structural studies in *Saccharomyces cerevisiae*, a basic module-based structure of the Mediator complex has been proposed (Guglielmi et al., 2004) and its homology was presumed and/or verified in many other organisms (Bourbon, 2008; Soutourina, 2018) This model dissects the Mediator complex into at least four associated modules: Head, Middle, Tail and CDK8/Kinase module. In *S. cerevisiae*, the complete complex is constituted from 25 and in human, from up to 30 subunit proteins (Figure 6). While Head and Middle modules are practically constant throughout more than a billion years of evolution, Tail module and some subunits are much more variable and are likely employed in specialized modes of signalling, *e.g.,* MED1 interacting with NRs is present mostly in animals, many of which use NRs (Figure 6).

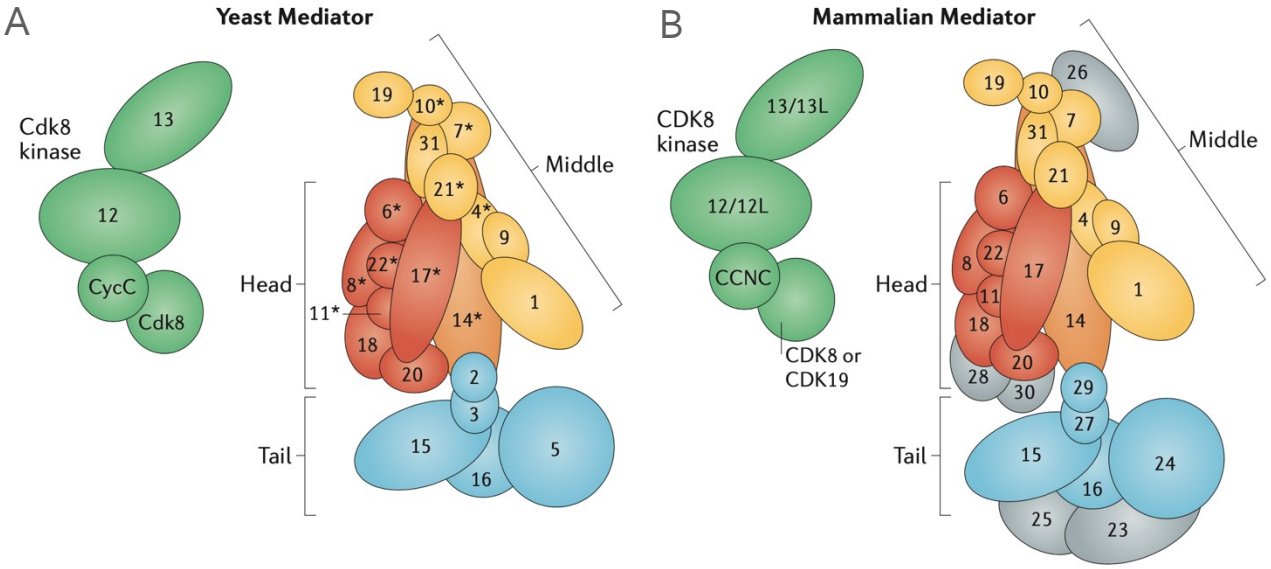


*Figure 6. Structure of yeast (A) and mammalian (B) Mediator complex with individual modules color-coded. Numbers denote the universal numbering of subunits. The placing of units 23, 25, 26, 28 and 30 absent from the yeast mediator is not experimentally proven (in grey). Asterisk marks subunits experimentally proven indispensable for viability in yeast. Adapted with permission from (Soutourina, 2018).*

While Head and Middle modules are considered the Mediator core and seem most important for Pol II and general TF-binding, Tail (and a part of Middle) module is a regulating dynamic module interacting with other signalling cascades. A stand-alone CDK8 module consisting in human from MED12, MED13 (or their paralogs), Cyclin C (CCNC) and Cyclin-dependent kinase 8 (CDK8) itself (or its paralog CDK19), possesses kinase activity and is implicated in downstream signalling and regulation of Mediator itself (Harper & Taatjes, 2018; Poss et al., 2013).

Structurally, the Mediator core structure is based on two critical intertwined subunits MED14 and MED17, each coordinating remaining subunits in Middle and Head module. The Middle subunit MED14 serves as a flexible backbone of the complex, connecting and correctly orienting the Head, Middle and Tail modules. It binds most subunits of the Middle module, the Head subunits MED17, MED6 and MED20 and the Tail subunits MED2 (MED29 in mammals) and MED15. The role of MED14 is not only structural. It is also highly flexible and spreads conformation changes that happen upon interaction of other subunits with binding partners and reverberates these changes throughout the complex, allowing by itself for the integration of multiple concurrent signals. On the other hand, MED17 central for the Head module. It makes contacts with most subunits in the Head nodule, but also MED4, MED7 and MED41 of the Middle module (Harper & Taatjes, 2018).

This described structure of "complete Mediator" is however likely not constant *in* vivo. Most notably, the CDK8 module is only transiently bound to Mediator and this interaction seems to be mutually exclusive with the interaction with Pol II. Upon binding of the CDK8 module, mammalian Mediator dissociates MED26 subunit (Ying Liu et al., 2001; Taatjes et al., 2002). It seems that the CDK8 module must be lost before Mediator can bind the pre-initiation complex (PIC) and activate Pol II, proposing an interesting model of regulation. This can be interpreted as an existence of various modular forms of Mediator, such as CDK8-Mediator and Pol II/(PIC)-Mediator.

Interestingly, outside the CDK8 module, Mediator does not have functional enzymatic activity. *S. cerevisiae* subunit Med5 possesses intrinsic acetyltransferase activity (Lorch et al., 2000) and Med8 participates on ubiquitin-ligase activity (Brower et al., 2002) but the main functions of Mediator do not seem to be exerted via classical enzymes. Rather, prevalent is a scaffolding function mediating contacts between sTFs, DNA, chromatin, GTFs and Pol II and transmitting allosteric effects via conformational changes in between all these molecules. Also, no DNA-binding of Mediator subunits is known to date, whilst the Mediator actions are sequence-specific. The binding of specific sequences of DNA and targeting Mediator to these sequences is therefore solely realised via sTFs (Poss et al., 2013).

Biochemically, a particularity of the Mediator subunit proteins is a high percentage of domains without defined secondary structure called Intrinsically Disordered Regions (IDRs) (Poss et al., 2013). The proportion of IDRs within Mediator subunits increases with the increasing complexity of an organism. This architecture endows Mediator with flexibility and variability of binding partners. In its function as a central integrator of incoming signals, these must be sensed in a way that allows for simultaneous stimulation and/or attenuation by many different proteins. In this regard, the flexible and complex structure of Mediator is perfectly adapted to bind various signalling proteins, most notedly ones binding specific sequences of DNA (Table 2). Individual subunits bind their typical binding partners and exert specific functions, all in concord with the allosteric effects of the remaining subunits and their binding partners.

One of the most important subunits in animals is MED1, a universal sensor subunit responsible for most of currently known interactions of Mediator. Its conserved structure contains two NR-boxes with canonical LxxLL motifs, allowing it to bind AF2 domains of a NR dimer. Most other regulatory subunits are a part of the Tail module, while MED1 is structurally a part of the Middle module and is much more variable than remaining Middle module subunits. It is only present in some organisms,

mostly animals. In yeast, it is the only Middle module subunit not required for the stability of the core complex and its knock-out mutants are viable (Mitsuhiro Ito et al., 2000).

Other than NRs, various different crucial pathways are transmitting their signals to Mediator as their final destination, where they are processed, integrated and a decision is made. Among these is the crucial p53 signalling of cell stress interacting with MED17, differentiation pathway Wnt/β-catenin ending in MED12 and innate immunity response signals transmitted from Toll-Like Receptors (TLRs) to MED23 (Soutourina, 2018). MED15 seems to be a specific subunit, performing also other roles outside its action in the Mediator complex (Malovannaya et al., 2011).

| MED1 | MED2 | MED3 | MED8 | MED12 | MED14 | MED15 | MED16 | MED17 | MED19 | MED21 | MED23 | MED24 | MED25 | MED28 | MED29 | MED31 | CDK8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TRα | Gcn4 | Gcn4 | Ace2 | RTA | GR | Smad2/4 | Dif | VP16 | REST | TRα | C/EBPβ | TR | VP16 | GRB2 | DSX | HSF | MYC |
| TRβ | | | | SOX9 | STAT2 | Smad3/4 | Gcn4 | p53 | | Tup1 | ESX | | RAR | | | | Hsf |
| VDR | | | | NANOG | HNF4 | NHR-49 | | ER | | | E1a | | HNF4α | | | | |
| RARα | | | | CTNNB1 | ERα | Oaf1 | | Hsf | | | ELK1 | | ERM | | | | |
| RXRα | | | | REST | PPARγ | Pdr1 | | Dif | | | Dif | | SOX9 | | | | |
| PPARα/γ | | | | G9a | SREBP1a | Pdr3 | | STAT2 | | | HSF | | ATF6α | | | | |
| ER | | | | Gli3 | | VP16 | | Gal4 | | | IRF7 | | | | | | |
| AR | | | | β-catn. | | Gal4 | | RXR | | | | | | | | | |
| GR | | | | AICD | | Gcn4 | | p65 | | | | | | | | | |
| HNF4 | | | | | | SREBP1a | | | | | | | | | | | |
| p53 | | | | | | | | | | | | | | | | | |
| BRCA1 | | | | | | | | | | | | | | | | | |
| NR4A | | | | | | | | | | | | | | | | | |
| FXR | | | | | | | | | | | | | | | | | |
| RORα | | | | | | | | | | | | | | | | | |
| AHR | | | | | | | | | | | | | | | | | |
| GATA-1 | | | | | | | | | | | | | | | | | |
| Pit-1 | | | | | | | | | | | | | | | | | |
| GATA-2 | | | | | | | | | | | | | | | | | |
| GABPα | | | | | | | | | | | | | | | | | |
| MYC | | | | | | | | | | | | | | | | | |
| POU1F1 | | | | | | | | | | | | | | | | | |
| 14-3-3 | | | | | | | | | | | | | | | | | |
| PGC-1α | | | | | | | | | | | | | | | | | |
| C/EBPβ | | | | | | | | | | | | | | | | | |

*Table 2 – DNA-binding proteins interacting with individual Mediator subunits. Modified from (Poss et al., 2013) under Creative Commons Attribution License. Mediator subunits are color-coded depending on their respective localisation in Mediator modules, as in Figure 6 (red for Head module, yellow for Middle module, blue for Tail module and green for CDK8 module). Among the interacting proteins are many NRs (red font).*

While most of the previously discussed subunits are conserved throughout the eukaryotic group of *opisthokonta*, which includes animals and fungi, some subunits, such as MED25, MED28 and

MED30 are more heterogeneous and absent from many phyla. For example, MED28 is absent in *S. cerevisiae*, but present in *metazoa*. This subunit is of particular interest in this thesis. All these variable subunits are intricate interactors and regulators, but not necessarily structural parts of the core Mediator complex.

Functions of Mediator are, as could be expected, manifold and complex. Its foremost and best studied function is the aforementioned promotion of transcription initiation by RNA Pol II. Transcription is initiated on TATA-boxes in target gene promoters. A TATA-box is bound by TBP, a part of the general transcription factor D complex (TFIID). Thereafter, remaining TFIIs and the RNA Pol II holoenzyme are recruited to the site, forming a pre-initiation complex (PIC). Mediator promotes PIC assembly by binding the Pol II itself, TFIIH (promoting CTD-phosphorylation), TFIIB, TFIID and TFIIF and stabilizing the whole complex, all while addressing this apparatus to the correct genomic site (Soutourina, 2018). MED26 subunit is important in RNA Pol II and TFII-binding (Paoletti et al., 2006; Takahashi et al., 2011). This subunit is absent from Mediator-CDK8 complexes, which might explain the mutually exclusive binding of Mediator by either CDK8 or the PIC (Ying Liu et al., 2001) and thus the CDK8-based regulation of Mediator function.

Mediator is also promoting transcription reinitiation (Yudkovsky et al., 2000), regulates transcription pausing and elongation (Allen & Taatjes, 2015), nuclear mRNA export (Schneider et al., 2015) and primes chromatin for transcription via direct interactions with histones and associated proteins (Soutourina, 2018). Mediator binds to histones and might displace them in front of the transcription machinery. More than that, Mediator brings together sTFs bound to linearly distant sequences and interacts with cohesion complexes, all of which results in DNA looping and eventually transcriptional memory (Kagey et al., 2010).

Inasmuch as the Mediator structure varies throughout evolution, it changes also during tissue specialisation and differentiation. While a relatively complete and even expression profile of Mediator subunits is present in stem cells, differentiated cells do not express all subunits in the same amount and some of these subunits are completely absent from certain differentiated tissues (D'Alessio et al., 2011; Deato et al., 2008). This is in concord with the differential expression profiles and various importance of different sTFs in specialized cells. The resulting variated structure of Mediator in specialised tissues of multicellular organisms suggests that (1) Mediator is concerned actively and passively in development and tissue specialisation; and that (2) alterations in the subunit composition of Mediator could lead to changes in cellular differentiation and to defects in its regulation.

Accordingly, human disease provides proof of these two points. Firstly, inborn defects in Mediator subunits MED12, MED17 and MED23 were associated with mental retardation and cerebral atrophy, in general developmental disorders (Soutourina, 2018). Secondly, acquired defects in Mediator function are of the utmost importance in many cancers. Indeed, subunits MED1, MED2/29, MED4, MED5, MED12, MED13/13L, MED14, MED15, MED17, MED19, MED23, MED28, MED30 and CDK8 have all been found to exert "driver" oncogenic effects in plurality of cancers. Acting as coactivators, many Mediator subunits function as pre-oncogenes and their up-regulation or more rarely activating mutation leads to neoplasms (Schiano et al., 2014).

### 1.3.2. Additional coactivators and corepressors

While Mediator is a dominant regulator of transcription initiation (and possibly elongation) by RNA Pol II, it seems that Mediator is not dominantly implicated in chromatin structure alteration. Rather, additional protein complexes possess internal or associated chromatin-modifying activity. These are self-standing integrating hubs of upstream signals, participating in a vast network of funnelling pathways (Figure 4).

One of the most central of chromatin-modifying coregulators is the cAMP response element binding protein (CREB) binding protein (CBP) and its homolog protein of 300 kDa (p300), as well as the p300/CBP-associated factor (P/CAF) (Chakravarti et al., 1996; Kamei et al., 1996). CBP/p300-P/CAF complex can bind NRs directly or via mediating proteins, most notedly proteins of the 160 kDa Steroid Receptor Coactivator (p160/SRC) family, that interact directly with the AF2 subdomain of NRs (J. W. Lee et al., 1995; Torchia et al., 1997; Voegel et al., 1996). This complex was first discovered in the context of its role in the cAMP pathway, but it is an effector in multiple other pathways including that of NRs. It possesses internal histone acetyltransferase activity, allowing for chromatin loosening and transcription progress.

Another important family of chromatin-modifying complexes implicated in gene expression regulation are the Switch/Sucrose Non-Fermenting (SWI/SNF) complexes, originally discovered in yeast (Cairns et al., 1994). Their presence in mammals was soon thereafter confirmed and an astounding structural and functional conservation of these complexes was established (Muchardt & Yaniv, 1993). The earliest described homologs of the SWI2/SNF2 proteins in mammals were called *brahma* and *brahma related gene-1* products (BRM/BRG1) and the remaining factors were termed BRM/BRG1-associated factors (BAFs). In human, the BAF complex contains between 4 and 17

subunits, constituted from 29 different genes (Hadidy & Uversky, 2019). In contrast to HAT complexes, SWI/SNF complex does not exert any histone-modifying activity, but rather inhibits ionic interactions between histones and DNA, loosening the wounding and promoting the displacement and/or sliding of histones before and during transcription (W. Wang et al., 1996). The exact interactions of these complexes with sTFs are currently not clear. However, BAF or SWI/SNF complexes have been shown to bind both NRs (Fryer & Archer, 1998) and p160/SRCs discussed above (Mckenna et al., 1998). Interestingly, recent research in this area concentrates on the seemingly significant role of SWI/SNF complexes in many cancers (Mittal & Roberts, 2020).

A third group of non-Mediator coactivators (fourth counting also the p16/SRCs) are E3-ligase complexes. The canonical role of these enzymes is sorting and targeting of proteins for proteasomal degradation. Interestingly, not only bind some E3-ligases to sTFs, most notedly NRs, but it has also been shown, that they directly mediate transcription activation. Specifically, a Homologous to E6-associated protein carboxy-terminal domain HECT domain family E3-ligases (Homologous to the E6-AP Carboxyl Terminus), most importantly the E6-associated protein (E6-AP) (Ramamoorthy & Nawaz, 2008), and another E3-ligase called hPRF-1 (Imhof & McDonnell, 1996) have been shown to mediate NR-dependent transcriptional activation. Curiously, the E3-ligase activity is not indispensable in this coactivation and the mechanism of action is likely dual (Ramamoorthy & Nawaz, 2008).

Transcriptional repression is likely an equally complex integrative network of signals with central role exerted in the case of most conventional subfamily 1, 2 and 3 NRs by conserved paralogs, Nuclear receptor corepressor 1 (NCoR1) (Hörlein et al., 1995) and NCoR-2 also known as Silencing Mediator of Retinoid and Thyroid hormone receptors (SMRT) (J. D. Chen & Evans, 1995). NCoR1 and 2 are paralogues with 50% conservation in primary structure. They consist of 2 (NCoR-1) or 3 (NCoR-2) C-terminal Receptor-Interacting Domains (RIDs) and in the N-terminus, of 3-4 Repression Domains (RDs) and a single deacetylase activation domain (DAD) (Astapova & Hollenberg, 2013). While RIDs interact with unliganded TRs and RXR, DADs bind to histone deacetylases (HDACs), specifically HDAC-3 and RDs were found to bind transducin binding protein like 1 (TBL1) and/or its paralogue TBLR1, as well as a supplementary G-protein Pathway Suppressor 2 (GPS2). HDACs, TBL1 and TBLR1 directly bind histones and exert chromatin modifying effects altering, mostly inhibiting, the access of Pol II to transcribed genes (Oberoi et al., 2011; Yoon et al., 2003).

## 1.4. Nuclear receptors of specific interest

I will now discuss in detail the NRs that are of particular interest in this thesis, the Retinoid X Receptors (RXRs), the Thyroid hormone Receptors (TRs).

### 1.4.1. The universal partner - Retinoid X receptor (RXR)

Likely the most ubiquitous, the best conserved and some of the best studied of all NRs are Retinoid X Receptors (RXRs). They are the defining members of the NR subfamily 2. Historically, RXRs were identified and cloned as supplementary retinoid receptors with different structure to the then known Retinoic Acid Receptors (RARs) (Mangelsdorf et al., 1990). They were among the first NRs to be structurally described, to have their ligand identified and their heterodimerisation principle clarified (Heyman et al., 1992; Levin et al., 1992). These discoveries stirred much scientific interest in the field of NRs, founding the field of molecular endocrinology, which is referred to as the RXR Big Bang (Evans & Mangelsdorf, 2014).

Evolutionarily, the presence of RXR orthologues can be traced back to the very beginning of multicellular animal life on Earth (Fonseca et al., 2020). Even more interestingly, their primary structure is extremely well conserved among organisms that diverged in evolution more than a billion years ago, *e.g.* the nucleotide sequence of an RXR orthologue in a very simple jellyfish *Tripedalia cystophora* is 61% to 68% orthologous to the human RXRα (Kostrouch et al., 1998). Retracing the common ancestor protein, one comes across the simplest *metazoan* presently living on Earth, the hallmark species of the phylum *Placozoa*, a flat saltwater amoeboid syncytium *Trichoplax adhaerens* (Novotný et al., 2017). Genome searches had suggested the presence of a sequence closely orthologous to human RXRα in *T. adhaerens*, with sequence conservation of 66%. This homology supersedes the homology of human RXRs to their human subfamily 2 paralogues HNF4 and COUP (Figure 7). In some animals, such as insects including *D. melanogaster*, no conventional RXRs have been found. Instead, a more distant RXR homologue called Ultraspiracle has been implicated in ecdysone signalling (Oro et al., 1990; Shea et al., 1990).

This conservation is however not only structural, but also functional. The *cnidarian* orthologue does not only bind 9-*cis*-retinoic acid with similar affinity and preference over all-*trans*-retinoic acid, but also binds to DNA on the consensus motif AGGTCA (see below) and it heterodimerizes with vertebrate TRβ (homologously to vertebrate RXR) on DR4 response elements (Kostrouch et al., 1998).

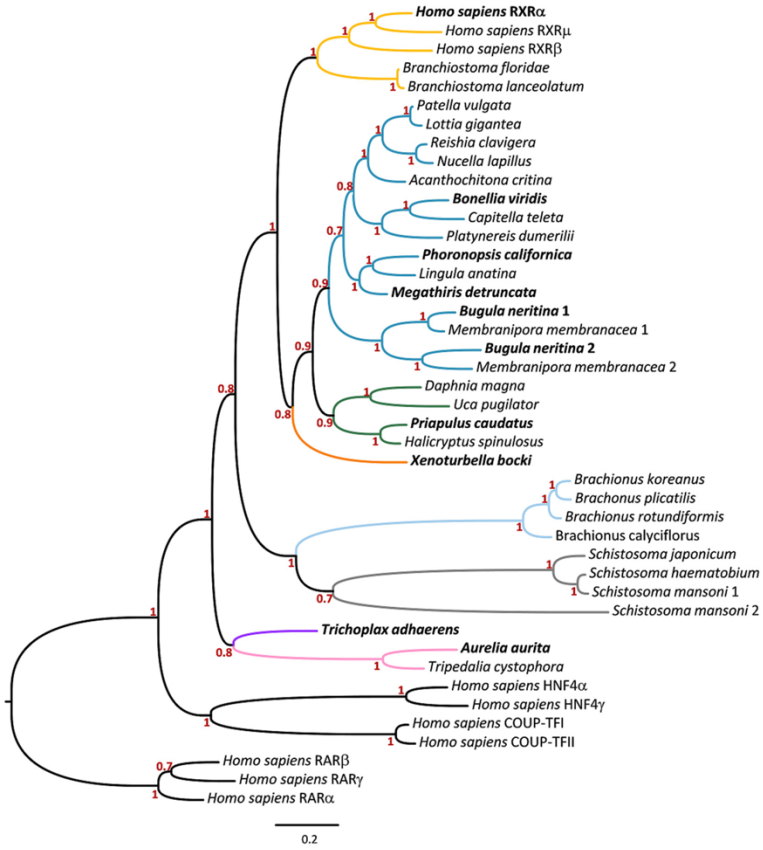


*Figure 7. RXR conservation in evolution determined by Bayesian sequence analysis. Adapted from (Fonseca et al., 2020).*

In mammals including human, three separate genes encoding three RXR paralogues are present: *RXRA* gene on the chromosome 9 encoding RXRα, *RXRB* gene on chromosome 6 with its protein product called RXRβ and *RXRG* gene on the chromosome 1 with its protein RXRγ. RXRα and RXRβ are very closely homologous and RXRγ is a somewhat more distant relative. All paralogues (and their several splicing variants) are all expressed relatively ubiquitously, RXRα being the dominant paralogue in visceral tissues, and RXRβ being abundantly expressed everywhere but in the viscera (Mangelsdorf et al., 1992).

Structurally, RXRs adhere to the consensus structural model of NTD with accessory AF1, DBD, hinge domain, and LBD. RXR DBD has similar structure to most other NRs with some particularities. Two Zinc-fingers prime the residues of two recognition helices placed on their C-termini to bind specifically the NR consensus motif in the major groove and a C-terminal (before hinge domain) residues constitute a dimerization interface called T-box. Unlike other NRs, the RXR exerts DNA-binding with lower affinity and specificity due to the recognition helices binding only three out of the 6 consensus base pairs (Zhao et al., 2000).

As is the case in other NRs, the LBD is an interacting hub responsible for most conditional functions of RXRs including ligand binding, dimerization, coactivator and corepressor binding. RXR LBD consists of 12 α-helices with 2 short anti-parallel β-sheets intercalated between helices 5 and 6. Its tertiary structure seems somewhat more straight-forward with individual helices more specifically dedicated to their respective roles than in other NRs, *e.g.,* the TRs (discussed below). The ligand-binding pouch is formed by residues of helices 3, 5, 7 and 11 and is much more flexible than that of NRs with dedicated ligands. Some residues in helix 5 can be repositioned, functioning as a pouch "hinge" (not to be confused with the hinge domain), fitting more voluminous ligands. The coregulator binding AF2 surface is also relatively simply formed by helices 3, 4 and 12, the last one being crucial for RXR function. Hydrophobic residues of these helices form numerous contacts with the consensus two-turn NR-box motif LxxLL on coactivators. The position of the helix 12 ensures specificity and stability of the bound, provided that this helix is positioned favourably, which depends on ligand-binding. In inactivated RXR LBD, helix 12 is not protruding from the LBD, destabilising coactivator binding, but providing sufficient space for a three-turn motif LxxxIxxxL, present on corepressors. Finally, the essential function of dimerization is mediated by helices 7, 8, 9, 10 and 11 (Dawson & Xia, 2012).

Interestingly, in most other NRs, ligand-binding alters the AF2-subdomain surface, especially fixing the helix 12 and permitting binding of coactivators. In RXR, however, ligand binding does not suffice. Only concomitant ligand binding and dimerization stabilises the helix 12, allowing corepressor dissociation and coactivator binding via the AF2 (Mika Ito et al., 2008). RXR is hence not capable of effectively transducing initiation signal on its own. This underscores the developed functional specialisation of RXR in dimerization.

While the AF2 and specifically helix 12 are important for coactivator binding, which is an almost universal trait of NRs, this subdomain in RXR also mediates interaction with corepressors. Different size of the two-turn sequence LxxLL of coactivators and the three-turn sequence LxxxIxxxL has been implicated in functional binding of antagonistic coregulators. Moreover, some ligand dependent corepressors like Dec1/2 and comodulators of PPAR and RXR 1 and 2 (COPR1/2) bind RXR via the coactivator motif LxxLL, while regulating transcription negatively (Cho et al., 2009; Flores et al., 2004).

The greatest particularity of RXRs is the uncertainty of their endogenous ligand. While most classical NRs of the subfamilies 1 and 3 have very well-defined ligands, RXRs bind a variety of endogenous ligands with various - generally weak - specificity. In this regard, they can be still considered orphan

NRs. In most cases, 9-*cis*-retinoic acid (9-*cis*-RA) is considered its natural ligand with the highest binding affinity. However, many authors argue that this substance cannot be natively detected in cells, unless its isomer all-*trans*-retinoic acid (ATRA) is artificially added to the cell culture medium (Mic et al., 2003; Wolf, 2006). Many other ubiquitous substances have been shown to interact with RXRs via their ligand-binding pouches, most notably unsaturated fatty acids. Out of these, the highest binding affinity has been shown for docohexaenoic acid (DHA) and arachidonic acid (AA) (Fan et al., 2003).

If RXRs have no high-affinity dedicated ligands, why are they considered the cornerstone NRs? In simple organisms directly chemically interacting with their surroundings, RXRs could have served as receptors of external stimuli. Yet, they still are indispensable in organisms as complex as human. There are several theories trying to explain their role in complex animals, but in general, all have to do with the dimerization of RXRs with other NRs.

Firstly, the DBDs of most NRs are structurally and evolutionarily rigid. Most NRs bind DNA specifically and with high affinity at the same nucleotide sequence motifs in the major grooves. This makes it difficult for individual NRs to distinguish their dedicated binding motif. LBDs are more plastic and can dimerize with other LBDs and these connections can more precisely determine the promoter specificity. Accordingly, as discussed above, response elements of NRs consist generally of two half-sites organized into direct repeats (DR), inverted repeats (IR), everted repeats (ER) or palindromic sequences. This serves as the unique selection filter determining which NR regulates the given gene. Indeed, conventional NRs bind DNA as monomers only with low affinity (Zhao et al., 2000). Hence, two NRs are needed for a specific and stable DNA-binding. In some cases, homodimers can be formed, as is the case of steroid receptors (subfamily 3), which are, however, regulated via a more recent nucleus-cytoplasm trafficking system. In the classical non-steroid receptors (subfamily 1), predominantly a stable and active heterodimer of one RXR with a second NR is formed.

Secondly, RXR induces allosteric changes in its heterodimerising partner NR, increasing coactivator binding by the latter even without directly binding the coactivator by itself. This has been termed the phantom effect of RXR (Son et al., 2008). No such effect is present upon homodimerization of subfamily 1 NRs. The increase in the partner's affinity to coactivator is also present when RXR is unliganded, but is stronger upon binding of 9-*cis*-RA. In this way, by using NR/RXR heterodimerization instead of NR homodimerization, nature exploits a modulation effect of RXR agonists on the classical hormonal regulation. Seeing as among RXR ligands are endogenous

unsaturated fatty acid, the content of which is cell-specific, this suggests that individual cells can adapt their response to globally acting hormones as the thyroid hormone and vitamin D.

RXRs heterodimerize with a whole spectrum of sTFs and are crucial for their function. Among the best studied are the interactions with NRs: RARs, PPARs, LXRs, CAR, TRs, VDR and Nurr-1. These heterodimers are formed specifically on gene promoters and enhancers depending only on the structure of the respective response elements. Some of the most important coordinating response elements have the structure of direct repeats. On DR-1 (direct repeat spaced by 1 random nucleotide), RXRs form homodimers and heterodimers with RARs, PPARs, HNF4s and COUPs. On DR-2, also RXR homodimers and heterodimers with RAR and PPAR were found. On DR-3, RXRs heterodimerize with VDRs. TRs, RARs and LXRs dimerise on DR-4 and RAR and certain orphan NRs bind RXRs on DR-5. In most heterodimers, RXR is binding the 5'-half site of the response element, one known exception being RAR/RXR heterodimer on DR-1 sites. More rarely, IR-1 (in case of CAR), IR-6, ER-6 and ER-8 (in case of PXR and SXR) are utilised (Dawson & Xia, 2012). Apart from these subfamily 1 NRs, RXRs bind many crucial transcriptional regulators such as NFκB, SMAD-2, circadian rhythm TFs, Oct-1, PML and many others (Lefebvre et al., 2010).

In terms of function, NR/RXR heterodimers can be divided into permissive, non-permissive and conditionally permissive (Dawson & Xia, 2012; Evans & Mangelsdorf, 2014). Permissive heterodimers such as RXR/PPAR, RXR/LXR and RXR/FXR dissociate corepressors and bind coactivators upon binding of a ligand to either the RXR or its binding partner and the effect is synergic. In non-permissive heterodimers RXR/TR and RXR/VDR, only TR and VDR ligands can mediate activation. Conditionally permissive heterodimers RXR/RAR and RAR/RXR are in between the two previous groups, they can get activated by RAR ligand binding, but thereafter the dimer function is strongly regulated by RXR ligands, whilst RXR ligands do not activate the dimer in the absence of RAR ligands (Figure 8).

These differential modes of permissive and non-permissive regulation seem to be structurally dependent on the interaction of the LBDs of both NRs. In order to function as permissive heterodimer, *i.e.,* to synergistically activate transcription via binding of both partners' ligands, the non-RXR partner's AF2 interacts with RXR helices 7 and 10, allowing transmission of allosteric information from one NR to another (Evans & Mangelsdorf, 2014; Gampe et al., 2000). More importantly, this ultimately results in changes in the N-terminal part of the binding partner's LBD, leading to dissociation of corepressors (Gampe et al., 2000).

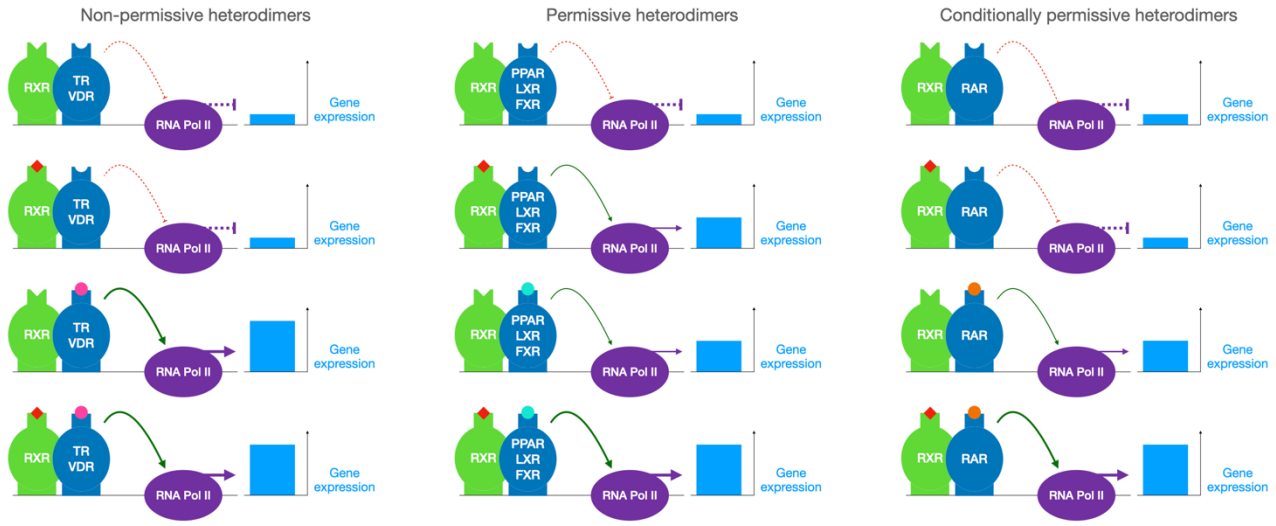


*Figure 8. Classification of RXR/NR heterodimers based on their function. Created based on data from (Dawson & Xia, 2012; Evans & Mangelsdorf, 2014).*

It seems, however that this model is oversimplified and permissivity is rather a continuous spectrum. In a cell type specific context, even non-permissive heterodimers can be regulated via the RXR ligands. *E.g.*, RXR/TR heterodimers are generally considered non-permissive, *i.e.,* only the binding of thyroid hormones can cause the dissociation of corepressors (NCoR1 and 2) and binding of coactivators. However, in pituitary cells, both RXR ligands and $T_3$ were able to activate transcription (Castillo et al., 2004). In somatic cells, binding of 9-*cis*-RA to RXR/TR dimer, causes weakening of coactivator binding (Putcha & Fernandez, 2009), as well as corepressor dissociation via its AF1 in the RXR NTD (D. Li et al., 2004). RXR acts therefore as a modulator of TR action, somewhat buffering the effect of $T_3$ and in some cells (pituitary), it might activate transcription.

An important structural particularity of RXR/NR heterodimers is the fact that while the 5' RXR interacts with both coactivators and corepressors via the AF2 subdomain, the partner NR uses AF2 only to interact with coactivators. Due to allosteric and space-occupation reasons in dimers, NRs such as TR and Nurr-1 bind their corepressors via a distinct N-terminal part of LBD via residues of the helix 1 (Apriletti et al., 1998; Lammi et al., 2008). This allows regulation by RXR ligands, as described above.

### 1.4.2. Metabolic coordinator - Thyroid hormone receptor

In vertebrates and especially in mammals, the thyroid hormone, 3,5,3'-triiodothyronine ($T_3$) is one of the most central regulators of development and metabolism. It is synthetized in the thyroid gland and secreted in an endocrine manner (to blood). Most vertebrate cells react to changes in the $T_3$ levels and possess thus the receptor apparatus capable of sensing this hormone. It is based on of Thyroid hormone Receptors (TRs), homologs of which are conserved throughout the phylum *Chordata* down to cephalochordates but absent in *Protostomia*.

In human, two distinct TRs are responsible for all physiological functions of $T_3$. These two TRs, denoted TRα and TRβ, are encoded by separate genes *THRA* and *THRB* located on chromosomes 17 and 3, respectively, with several tissue-specific splicing variants of both (Benbrook & Pfahl, 1987; Pfahl & Benbrook, 1987; Sap et al., 1986; Weinberger et al., 1986). These two paralogues have differential tissue expression profiles with diverse relative importance of the physiological effects exerted by the individual TRα and TRβ in various tissues. TRα is the dominant TR expressed in neural tissue and in the heart, while TRβ has a more equally distributed expression in most human cells (Akihir Sakurai et al., 1989). This suggests polarized roles of TRα in development and of TRβ in metabolism. Importantly, TRβ is solely responsible for the hypothalamic and pituitary feed-back loop discussed below (Weiss et al., 1997).

TRs are typical subfamily 1 NRs with the standard structure described above (Figure 4). Between TRα and TRβ, a high degree of homology is seen in both the DBD (88%) and the LBD (86%), whilst no homology is present in the NTD (Apriletti et al., 1998). Their ligand-binding domain is constituted of 12 α-helices and 4 β-sheets packaged into a globular tertiary structure. This structure was first resolved by X-ray crystallography in 1995 on rat TRα (Wagner et al., 1995). The core of the LBD is formed by the ligand cavity, on the formation of which most of the LBD contributes. Helices 2, 3, 5, 6, 7, 8, 11 and 12 and a loop between sheets 3 and 4 all participate in the formation of a hydrophobic cavity with a volume of 600 $Å^3$. $T_3$ constitutes a part of the receptor, coordinating its folding (Wagner et al., 1995). Helices 9 and 10, along with the helix 11 are responsible for homo- and heterodimerization and helix 1 is important in corepressor binding (Apriletti et al., 1998; Wagner et al., 1995). Helices 3, 5, 6 and 12 mediate also, along with the remaining helix 4, the crucial coactivator binding function by forming an AF2 subdomain, which binds the consensus LxxLL sequence of coactivators (Figure 9) (Apriletti et al., 1998).

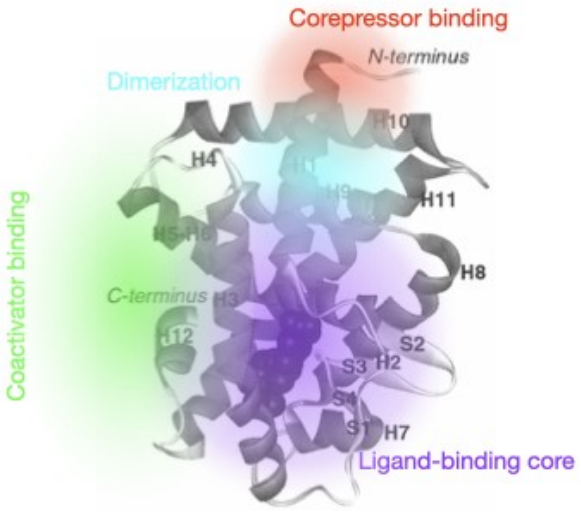


*Figure 9. Structure of TR LBD resolved by X-ray crystallography with noted functional regions. Based on the original structure reported (Wagner et al., 1995), edited as in (Apriletti et al., 1998) and adapted with permissions.*

TRs bound to their thyroid hormone response elements (TREs) (conventional 5'-AGGTCA-3' hexad sequences) form either homodimers (TRα/TRα and TRβ/TRβ), interindividual heterodimers (TRα/TRβ) or most importantly heterodimers with RXRs on DR4 elements. Helices 9, 10 and 11 in LBD alongside the D-box subdomains of DBDs of the TRs are responsible for these interactions. The exact mechanism of the dimerizations is not completely understood. Ligand binding seems to alter dimerization capacities, likely via allosteric reverberations in the LBD leading to altered affinities of the helices 9, 10 and 11 to individual binding partners. While $T_3$-unbound TRs might bind TRE as homodimers, RXR/TR heterodimers are preferred upon $T_3$-binding (Apriletti et al., 1998).

TRs are ambiguous regulators of gene expression, based of hormone binding and likely, cell-specific context (discussed below). Most genes are regulated positively by $T_3$, *i.e.,* hormone activates their expression. In the case of these positively regulated genes, inactive, $T_3$-unbound TRs act as negative regulators of gene expression on their target genes (blocking transcription to a sub-native level) and in $T_3$-bound state, they act as positive regulators. However, there are also negatively regulated genes, where TRs suppress specific gene transcription upon $T_3$-binding (Koenig, 1998) (Figure 10). This is discussed in detail below (1.4.3).

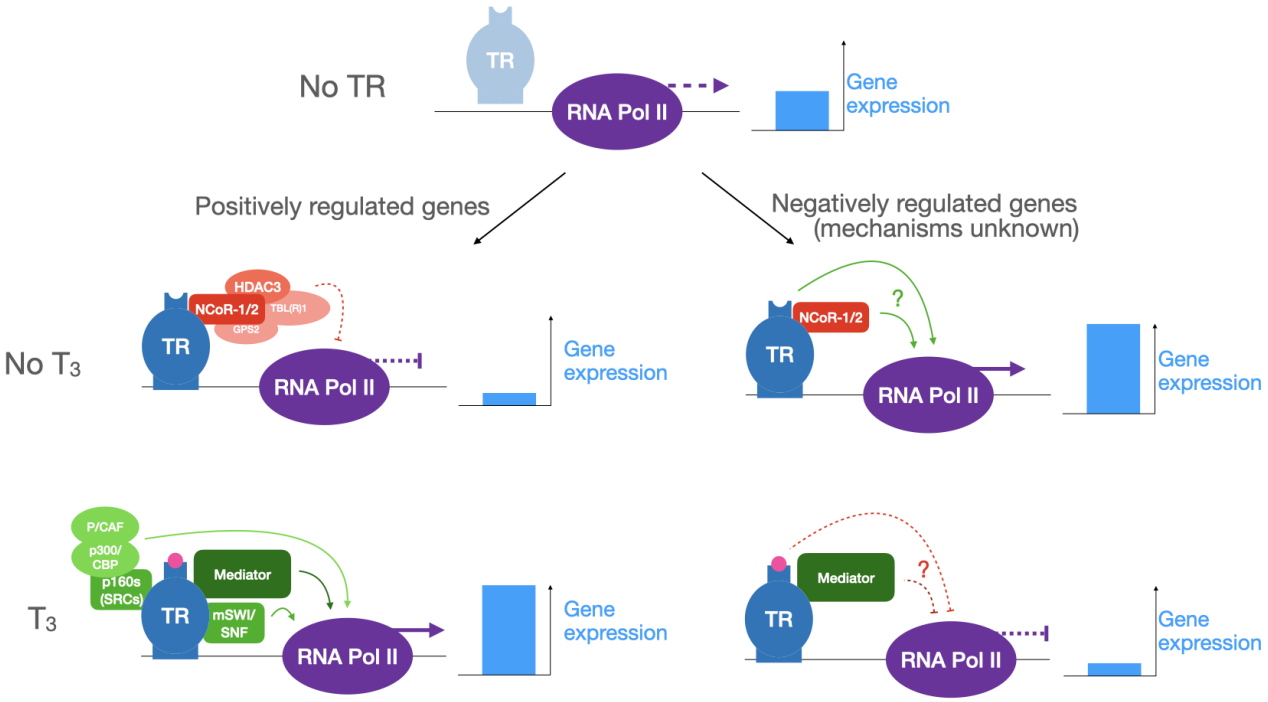


*Figure 10. Mechanisms of TR regulation on positively and negatively regulated genes. Most important proteins and complexes participating in this regulation marked in-picture. The mechanisms of regulation of negatively regulated genes not being fully understood, the figure shows one of proposed consensus models. Created based on data from (Clifton-Bligh et al., 1998; Kaššák et al., 2017; Ortiga-Carvalho et al., 2005; Akihiro Sakurai et al., 1990; Sasaki et al., 2018; D. Wang et al., 2010).*

In the absence of hormone, TR/TR homodimers and RXR/TR heterodimers are localized to TREs dispersed along the human genome, binding to corepressors, most notedly the NCoR1 and NCoR2 (SMRT). NCoRs bind directly or indirectly to chromatin-modifying enzymes, inhibiting transcription to a sub-native level.

Upon $T_3$-binding, TRs dissociate NCoRs, TR/TR homodimers may be replaced with RXR/TR heterodimers and they bind coactivators via their AF2 subdomain, *i.e.* the lateral surfaces of helices 3, 4, 5 and 6, with helix 12 serving as a hydrophilic clamp, securing the bond. The consensus coactivator motif binding to AF2 consists of three leucines spaced by two random amino acids (LxxLL) (Apriletti et al., 1998). This motif is present on a great number of coactivators, of which the most important are the Mediator subunit 1 (MED1), p160/SRC family proteins, p300/CBP and P/CAF complexes, BAF *i.e.,* SWI/SNF complexes and E3-ligases. These complexes exert, in turn, alterations in chromatin structure and stimulate directly and indirectly Pol II to initiate and carry on transcription,

as discussed in section 1.3.2. (Chakravarti et al., 1996; Coulthard et al., 2003; Kamei et al., 1996; Loinder & Söderström, 2004; Ramamoorthy & Nawaz, 2008; W. Wang et al., 1996).

### 1.4.3. Negatively regulated genes in TR-dependent regulation

Not all genes are regulated in the above-described manner, *i.e.,* positively. On a fraction of promoters, TRs employ unknown mechanisms to stimulate transcription in absence of $T_3$ and repress it in its abundance. This allows cells to suppress actively gene expression of genes, which are not to be transcribed in the given scenario of hormonal stimulation. This is the most direct feedback mechanisms and seems to play an important role in thyroid hormone regulation in human.

Despite that, this inverse regulation of gene expression remains elusive and very little is known about its mechanisms. Inside a single cell nucleus, there must be present mechanisms of positive and negative regulation exerted by the same signal (presence of hormone). Since TRs are the only sensors of $T_3$, these mechanisms must exploit the same receptors, that must act inversely on certain genes in the same nucleus. Moreover, it seems that these regulations are tissue specific.

To accomplish this, in theory, TRs could employ one of three mechanisms: either (1) different TR isoforms and/or different dimerizations invoke differential coregulator binding on negatively regulated promoters, *i.e.*, unliganded TRs bind coactivators and liganded TRs corepressors; or (2) the same TR dimers bind to promoters of positively and negatively regulated genes, but other signals modify coregulator binding, also binding coactivators when unliganded and corepressors when bound to $T_3$; or finally (3) $T_3$-dependent interactions of TRs with coactivators and corepressors are not inversed (*i.e.* liganded TRs bind coactivators and unliganded corepressors), but the ultimate action of coregulators is opposite, where coactivators such as Mediator silence, and corepressors activate gene expression (Sinha & Yen, 2000).

Current knowledge is in favour of the last mode of function (3). On the promoters of negatively regulated genes, corepressors seem to mediate basal transcriptional activation upon contact with unliganded TRs (A. N. Hollenberg et al., 1995; Krishna et al., 1989; Ortiga-Carvalho et al., 2005; Tagami et al., 1997; D. Wang et al., 2009, 2010). Inversely, coactivators may silence gene expression of the negatively regulated genes in the presence of $T_3$ (Tagami et al., 1997, 1998). Some works suggest that this regulation is independently performed in a *cis* fashion, based on special classes of negative response elements (Bodenner et al., 1991; A. N. Hollenberg et al., 1995). However, this has been contested and the whole mechanism remains elusive. Also, the situation is rendered even more

complex by the necessity of supplementary activators such as GATA2 and might rely partially on the isoform TRβ2 (Matsunaga et al., 2015; Matsushita et al., 2007). Nevertheless, this does not interfere with the basic concept of negative regulation by TRs.

Seeing as the LBD of TRs is very well studied and individual functions can be precisely mapped to its helices, subdomains and even amino acid residues, studies of structure-function relationship could also explain the mechanisms of the negative regulation by TRs. Structure-function studies are routinely performed by introducing mutations to protein-coding genes and following the resulting phenotype. However, TRs are crucial for development and metabolism and many mutations in their genes are lethal. This makes it excessively complicated to study TRs structurally. A unique opportunity to study the functional determination of individual structural units of TRs is offered by observing phenotypes of mutations naturally occurring in the best studied animal of all, human. Indeed, various mutations in *THRA* and *THRB* genes cause distinct forms of the syndrome of Resistance to Thyroid Hormone (RTH). Reverse observational studies of these patients present valuable information on structure-function relationships inside the TR proteins. This approach has been used previously to study negative regulation by TRs (Clifton-Bligh et al., 1998; Tagami et al., 1998), but strenuous molecular binding analyses and reporter assays had been required due to the lack of complete functional mapping of the TR β LBD. Presently, a mere functional analysis of the final phenotype coupled with *in silico* analysis of the impacted residues provides sufficient information about the importance of TR functions in the negative regulation.

### 1.4.4. Syndrome of resistance to thyroid hormone (RTH)

To understand the pathogenesis of the RTH syndrome, one must consult the physiological regulation of thyroid hormone action, as well as the structure of TR genes.

While the biologically most active form of the thyroid hormone is $T_3$, the most prevalent form is thyroxin ($T_4$). $T_4$ is converted into $T_3$ and other inactive forms by cytoplasmic deiodinases in target cells (Braverman et al., 1970; Sterling et al., 1970). Both $T_4$ and $T_3$ are physiologically synthetized from tyrosine by a complex pathway of endo- and exocytosis in the thyroid gland (Bernier-Valentin et al., 1991; Dunn et al., 1996; Frati et al., 1974; Nakagawa & Ohtaki, 1985). This pathway is under intricate coordinating regulation of thyrotropin, also known as thyroid stimulating hormone (TSH). TSH is a glycoprotein hormone secreted by thyrotrope cells in the anterior pituitary (adenohypophysis). It consists of two protein subunits α and β. While the α subunit is a 93-amino

acid peptide chain jointly used by the TSH, the luteinizing hormone (LH), the follicle-stimulating hormone (FSH) and the human choriogonadotropin (hCG), the β subunit is unique for TSH (Magner, 2014). The expression of both α and β subunits are regulated by transcriptional activation by a supraordinate Thyrotropin-Releasing Hormone (TRH). TRH is a tripeptide hormone produced by cleaving of a precursor polypeptide in the parvocellular neurons of the paraventricular nucleus of hypothalamus (Figure 11) (Mullur et al., 2014).

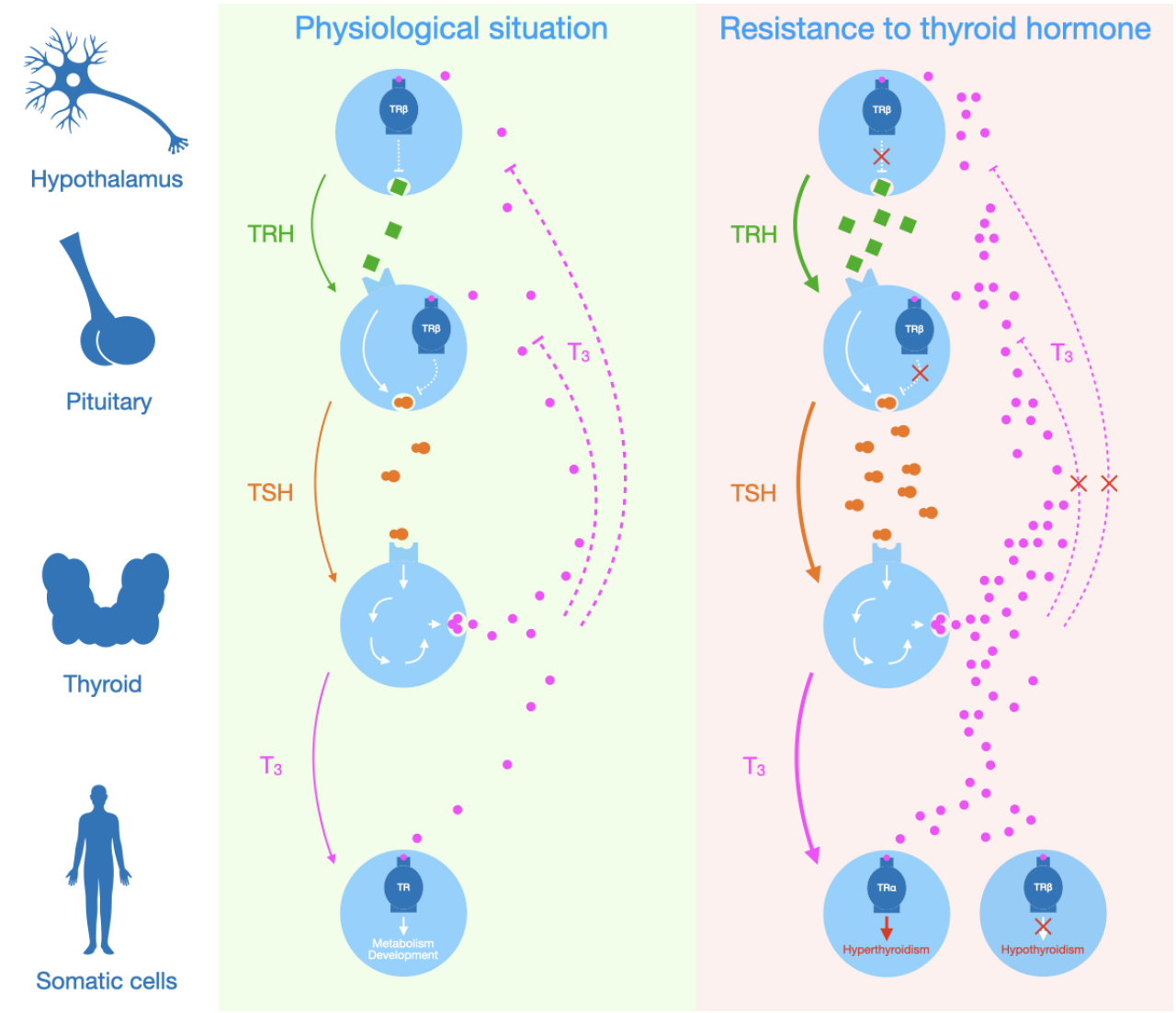


*Figure 11. The conventional Hypothalamus-Pituitary-Thyroid axis under physiological conditions and in the syndrome of resistance to thyroid hormone β. Created based on data from (Dumitrescu & Refetoff, 2013; Pappa & Refetoff, 2018; Persani & Campi, 2019).*

Importantly, the production of both TSH (α and β) and TRH (precursor polypeptide) are suppressed by direct transcriptional silencing mediated by $T_3$-bound TRβ. The discussed negative regulation by TRs is crucial in this step, as all three genes are regulated negatively, while most other genes in these

cells are regulated positively by $T_3$. A restrictively expressed TRβ2 splicing variant seems indispensable in this negative regulation (Dupré et al., 2004). Hypothalamus-produced somatostatin serves as negative regulator of TSH production, as well (Ampofo et al., 2020).

The aetiologies of RTH are multiple. Rare cases of families with complete homozygous deletion of *THRB* gene have been described (K. Takeda et al., 1992) and only recently, a handful RTH cases caused by defects in *THRA* gene (RTHα) have been discovered (C. Moran et al., 2017). Most known cases of RTH are however caused by miss-sense single-nucleotide polymorphisms (SNPs) in the *THRB* gene (RTHβ). These are of particular interest in the structure-function studies of TRβ, as a role of individual amino acid residues can be determined and studied *in vivo*, providing an uncontested functional resolution.

The *THRB* gene on chromosome 3 consists of 10 exons spaced by long introns. The LBD is encoded by exons 7, 8, 9 and 10. Missense SNPs leading to the RTHβ are concentrated to these four terminal exons and no mutations have been described in the sequence encoding NTD or DBD. Most of the mutations are localized to one of three clusters ordered by the frequency of individual SNPs among patients: *cluster 1* at the far end of exon 10, corresponding to the helices 11 and 12; a broader *cluster 2* in the middle of exon 9 and thus also the LBD; and a *cluster 3* at the junction of exons 7 and 8, localizing to the C-terminus of hinge domain and N-terminus of the LBD (around helix 1 of the LBD). These clusters are interspaced by so-called cold regions, where no mutations have been found in RTHβ patients (Figure 12) (Dumitrescu & Refetoff, 2013; Persani & Campi, 2019).

RTHβ is a hereditary syndrome with autosomal dominant (AD) transmission. It is caused by heterozygous germline mutation exerting a dominant negative effect (DNE) on the TR regulation (Akihiro Sakurai et al., 1990). The mere lack of one functional allele does not explain the phenotype, but rather a deleterious interference of the defunct TR is responsible for all symptoms. Several explanations for this DNE have been proposed: homo- or hetero-dimerization of the mutated TR with normal TR or RXR blocks the response element site, but the dimer (1) cannot respond to activation by $T_3$ (Akihiro Sakurai et al., 1990); (2) cannot dissociate from corepressor binding (Yoh et al., 1997); or (3) cannot bind coactivators (Collingwood et al., 1998).

The prevalence of the syndrome is estimated at between 1:18 750 (Vela et al., 2019) and 1:40 000 (LaFranchi et al., 2003) but is likely very underdiagnosed seeing as it is not discovered by perinatal TSH screening. Clinically, RTHβ patients experience a relatively inhomogeneous *tableau* of symptoms combining signs of hyperthyroidism, hypothyroidism and biochemical disturbances.

Among the most common presentations are diffuse or multinodular goitre, tachycardia with palpitations, attention deficit and hyperactivity disorder (ADHD) and predisposition to psychiatric illnesses, paediatric growth retardation and failure to thrive, *etc*. Biochemically, RTHβ patients present elevated free $T_4$ ($fT_4$) and $fT_3$ levels with unchanged $fT_4/fT_3$ ratio and upper normal range or slightly increased TSH levels (Dumitrescu & Refetoff, 2013; Pappa & Refetoff, 2018; Persani & Campi, 2019).

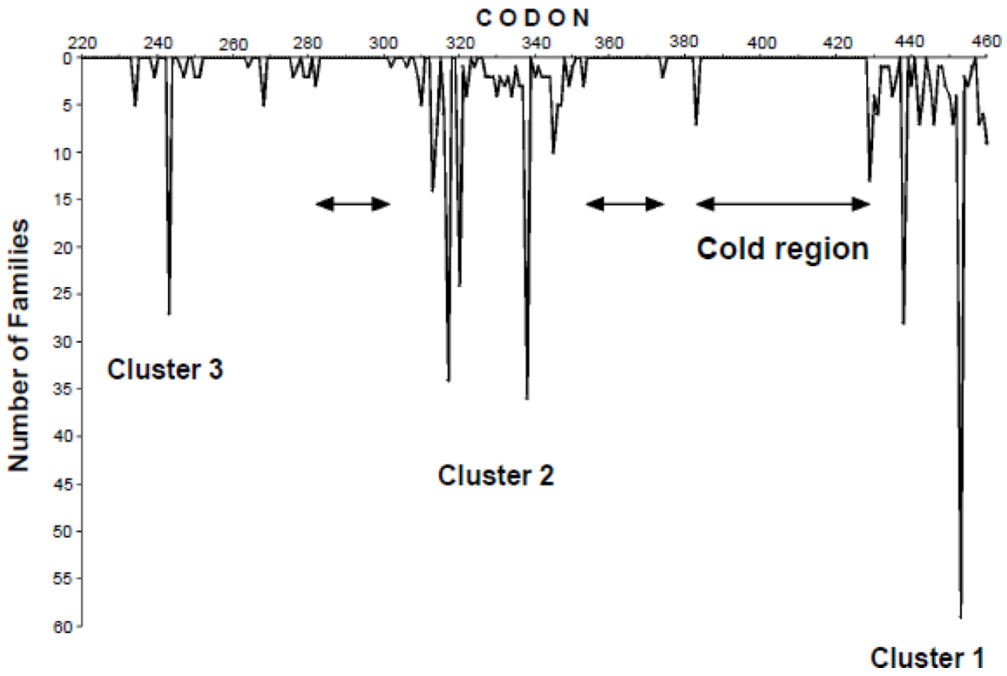


*Figure 12. Clusters of mutations causing RTHβ with their reported frequency. Reused with permission from (Dumitrescu & Refetoff, 2013).*

The pathogenesis depends on the dominant negative effect of mutated TR disturbing normal TRβ function. In most cells dependent of TRβ or mixed TRα and TRβ signalisation, dysfunction of TRβ results in lack of transcriptional activation of the predominant positively regulated genes. In hypothalamus and anterior pituitary, negative regulation by TRβ is normally curbing the production of TRH and TSH. With the dysfunctional TRβ signalling, this negative feedback loop is disinhibited and TRH and TSH are produced in a surplus. This stimulates thyroid to increase production of $T_3$ (and $T_4$), the level of which is augmented, but still cannot impede the TRH/TSH production. Resulting increased levels of thyroid hormones continue to up-regulate transcription in cells where TRβ interference is minimal, *i.e.,* in cells relying under normal circumstances on TRα mediated $T_3$ signalling. The resulting somatic phenotype is a mosaic of hypothyroid and hyperthyroid symptoms in different tissues (Figure 11) (Dumitrescu & Refetoff, 2013).

Clearly, the defining moment of this pathogenesis is the impaired negative regulation in hypothalamic neurons and thyrotropes of anterior pituitary. By studying individual mutations, one can functionally dissect the specific amino acid residues, LBD helices and subdomains of TRβ.

## 1.5. Study strategy and reasoning

The work described in this thesis has taken for an aim to contribute to the current understanding of biological processes implemented during transcriptional regulation by NRs and their cofactors, with an ultimate goal of applicability in medical practice, specifically in oncology.

Firstly, I focus on the most central regulator of metabolism and ontogenesis that is often dysregulated in cancer, the thyroid hormone receptor (TR) (Burke & McGuire, 1978; Chan & Privalsky, 2009; R. N. Chen et al., 2008; Chung et al., 2015; García-Silva & Aranda, 2004; Kamiya, 2002; Lin et al., 1999; Poplawski & Nauman, 2008; Puzianowska-Kuznicka et al., 2002; T. Wang et al., 2016). Of specific interest is the mechanism, which reverses the consensus function of TR into the negative regulation, as this could be ultimately used in targeted suppression of aberrant TR action in some cancers. I study the molecular reverberation of a newly discovered mutation in TRβ in patients with the syndrome of resistance to thyroid hormone β (RTHβ).

Secondly, I participated in an effort to study the indispensable dimerising partner of TRs and a protein with a dual role in carcinogenesis, the RXR (Casas et al., 2003; Halstead et al., 2017; Matsushima-Nishiwaki et al., 1996; Ye et al., 2019). My special interest was the regulatory potential of the primordial RXR in homolog in an organism at the basis of animal evolution, *T. adhaerens*. We were able to provide structural information in the mechanistic model of TR/RXR-based negative regulation.

And thirdly, I venture to clarify and amend the previous discrepancies regarding the most important coactivator, the Mediator complex. Specifically, my colleagues and I study a peculiar Mediator subunit MED28, implicated in negative regulation by Mediator (Beyer et al., 2007), as well as in carcinogenesis in several cancers (C.-Y. Huang et al., 2017; M. F. Lee et al., 2011, 2016). We examine the reasons for this subunit's apparent lack of structural and functional conservation in a model organism *C. elegans*, where otherwise the Mediator complex is relatively well conserved. Thus, we provide the missing piece of puzzle of the Mediator conservation and we correct an improper previous presumption, that MED28 is absent in this important model organism. We analyse its important role in development and its possible implication in the negative regulation.

Put together, these studies allowed me to support three principal conclusions of this thesis: (1) conservation of the NR-Mediator regulation of gene expression; (2) mechanistic model of the negative regulation by NRs; and (3) exploitability of animal models in the studies of NR/Mediator-mediated carcinogenesis.

### 1.5.1. Rationale of reverse genetic studies on TRβ

Arguably, human is the best studied organism on the planet in both the biochemical and clinical perspective. Patients are followed often throughout their lifetimes and the methods of clinical examination and paraclinical investigations have been developing for millennia. Moreover, most of current research in life sciences is conducted with the interest of ultimate applicability in humane medicine. As such, studies of molecular biological mechanisms in human are directly applicable and do not necessitate interpolation and validation across species. However, it is obviously not ethically acceptable to conduct interventional experiments in human subjects and our scientific *repertoire* is limited to observation and therapeutic essays. We may, on the other hand, search for naturally occurring genotypical alterations, polymorphisms, mutations and epigenetic alterations and correlate them phenotypical particularities of human probands.

This approach has been used to locate causal mutations of many previously unexplained genetic syndromes with monogenic heredity and lead to a clarification of the aetiology of numerous diseases. At the same time, it allowed clarification of various physiological pathways and mechanisms. However, it is important to realise that only a fraction of all human proteins and their parts can be studied in this way. A great portion of mutations be they larger deletions, rearrangements or even simpler substitutions of one nucleotide for another, result in lethal embryonic phenotype, the bearers are never born and these mutations do not occur in nature. Most of the remaining non-lethal genetic alterations do not present themselves as clearly characterizable diseases, seeing as they do not disturb – or not significantly – the physiological mechanisms. These are mostly mutations in non-coding regions of DNA, same-sense SNPs and other non-critical alterations. They do not introduce a negative selection and they may randomly spread in populations. Some of these may jointly contribute to pathogenic traits, establishing a pattern of polygenic heredity. Most of presently common diseases are inherited in this manner, *e.g.,* arterial hypertension, type II diabetes mellitus, most of familiar cancer predispositions, psychiatric diseases, *etc*. Only the very small reminder of aberrations present themselves with a clear phenotypical change, where studying the relationship between genotype and

phenotype can convey mechanistic understanding of the underlying processes (Cummings et al., 2019) (Figure 13).

Conveniently, the RTHβ falls into a category of diseases with clear monogenic heredity and has a distinct biochemical phenotype that can be well quantified: disturbance of the hypothalamus-pituitary-thyroid (HPT) feedback axis.

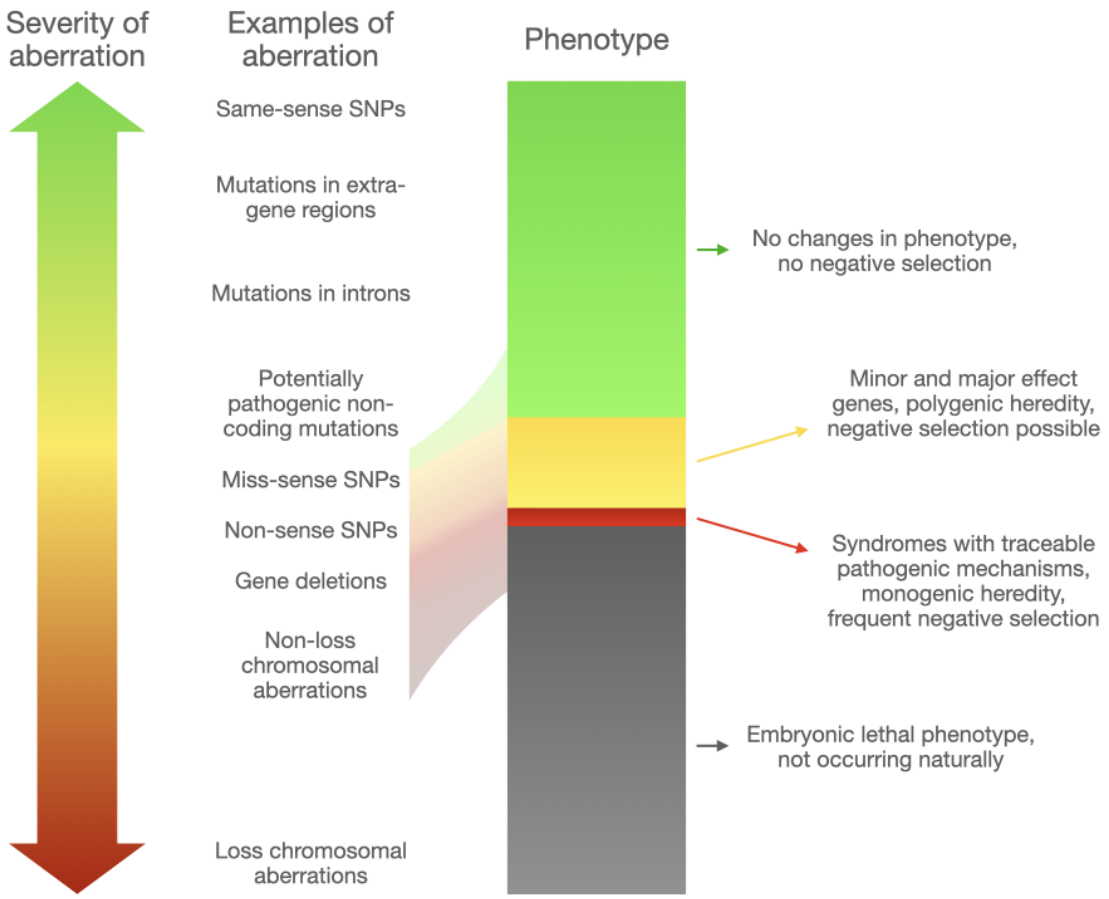


*Figure 13. Illustrational theoretic diagram of naturally present mutations and their phenotypical presentation. Only a fraction of all mutations (red) are sources of clearly defined pathologies that can be used to study molecular mechanisms. Created based on data from (Cummings et al., 2019).*

We proposed to search for previously undiscovered mutations in the *THRB* gene by looking for patients with symptoms compatible with the syndrome of resistance to thyroid hormones (RTHβ). Seeing as the syndrome is based on a dominant negative effect of the mutated TRβ receptor, patients presenting the dysregulation of the HPT axis must possess a semi-functional receptor with maintained DNA-binding and dimerization capacities, but insufficient activation upon $T_3$-binding. We have ventured to map the discovered mutations onto the well-established structure of the TRβ LBD and to

assess particularly mutations causing structural alteration of the coactivator- and corepressor-binding surfaces. In contrast to the mutations impairing ligand binding, these mutations could help distinguish the subdomains and thus the coregulators responsible for the various impaired functions.

### 1.5.2. Principle and rationale of evolutionary studies of RXR in *T. adhaerens*

Targeting of NRs including TRs to the genes they regulate is solely determined by the organisation of the target gene promoter and thus by NR dimerization. Therefore, the signal for the reversal of TR-mediated gene expression activation to repression can only come through differential dimerization with its partner NR. Indeed, there are distinct dedicated negative thyroid hormone response elements (nTREs) in negatively regulated gene promoters that in some cases revert regulation by TRs, although the situation is likely much more complex (Sasaki et al., 2018). Moreover, gene expression regulation by $T_3$-TRs is evolutionarily relatively recent. It can be presumed that negative regulation by NRs is evolutionarily more ancient than the appearance of TRs.

For both reasons, my focus was aimed at the dimerising partner of TR, Retinoid X Receptor (RXR), RXR is much more evolutionary conserved than TR and is of crucial importance in the TR-mediated negative regulation (T. Takeda et al., 1997). The simplest known animal, *Trichoplax adhaerens*, was selected to study the structure and function of its RXR.

### 1.5.3. Rationale of studies of MED28 in C. elegans

In its role as a coactivator, the Mediator's consensus role is gene expression activation via direct interaction with Pol II as well as through other mechanisms described in section 1.3.1. As such, it is also of crucial importance in TR-mediated positive gene regulation. An important question is whether Mediator could also function as a corepressor, among other things, in the negative regulation by thyroid hormone that seems dependent on coactivator binding and the repressing function of coactivators. What is surprising when considering the sheer complexity and sophistication of Mediator and the quantity of nuclear processes it is implicated in, to date, only 2 isolated Mediator subunits were shown to harbour repressive activity: MED3 (Papamichos-Chronakis et al., 2000) and MED28 (Beyer et al., 2007).

Specifically, MED28 seems to coordinate a submodule along with MED6, MED8 and MED18 inside the Head module that acts as a transcriptional repressor. This function has been shown in smooth

muscle cells, where MED28-dependent gene repression counteracts smooth muscle differentiation (Beyer et al., 2007).

MED28 has been first derived from endothelial cells (hence termed Endothelial derived Gene 1, EG-1) (C. Liu et al., 2002), and only later identified as an important animal Mediator subunit (Sato et al., 2004). This 24kDa protein is localised to the Head module of *metazoan* Mediators. It has been found to interact with actin cytoskeleton, Growth factor Receptor Bound protein 2 (Grb2) and Neurofibromatin/Merilin on the cell membrane (Wiederhold et al., 2004), with a variety of Src-kinases (M. F. Lee et al., 2006) and to mediate effects of the β-catenin signalling pathway (M. F. Lee et al., 2016). This allows transduction of a great variety of cytoplasmic signals and mechanical stimuli to Mediator, ultimately regulating gene expression.

Importantly, cells overexpressing MED28 in laboratory conditions retained multipotent stem-cell character and upon its knock-down, differentiation appeared (Beyer et al., 2007). This effect was dependent on the negative regulation of differentiation-promoting genes by MED28, suggesting a causal connection of the negative regulation by Mediator – a coactivator – and carcinogenesis. Accordingly, MED28 is highly expressed in breast cancer, colorectal cancer and prostate cancer. Up-regulation of MED28 in breast cancer is associated not only with cell-cycle deregulation but also with epithelial-mesenchymal transition (EMT), which is required for invasion and metastatic spread (M. F. Lee et al., 2011). More recently, it has been shown that this EMT induction happens via alteration of the NFκB inflammatory pathway (C.-Y. Huang et al., 2017). It seems therefore that MED28 is a central pre-oncogene responsible for numerous aspects of defective regulation in cancer.

This very interesting subunit does not have an orthologue in *S. cerevisiae*, making its study more difficult. One convenient model organism that had had a described MED28 homologue was *Caenorhabditis elegans*. In the *C. elegans* genome, a gene in the locus W01A8.1 had been annotated by large-scale genomic studies as the *MED28* orthologue called *mdt-28* (Bourbon, 2008). Strikingly, in contrast to other species, the protein product of *W01A8.1* does not localize to the nucleus but rather is a dominant resident protein of cytoplasmic lipid storage organelles called lipid droplets (LDs) (Zhang et al., 2012).

# 2. Materials and methods

Underlining the evolutionary approach in my attempts to address this thesis' scopes, my colleagues and I employ some of the most powerful and intriguing model organisms, *Caenorhabditis elegans* and *Trichoplax adhaerens*, as well as a medical translational approach to indirectly study molecular biology in human.

## 2.1. Genetic analyses of naturally occurring mutations in *H. sapiens* TRβ

### 2.1.1. Approvals, patient accrual and selection

In order to recruit patients with the very rare syndrome of resistance to thyroid hormone β (RTHβ), we initiated a cooperation with clinical endocrinology experts specialised in the thyroid hormone endocrinopathies. We prepared a study plan, applied for and obtained an ethics committee approval for the study with reference: "Approval of June 21, 2012, project title: Genetic and Molecular Characterization of the Regulation by Thyroid Hormone, Chairman of the Committee Josef Šedivý".

Patients and their families were addressed from associated endocrinologists and paediatric endocrinologists from a larger catchment area of Middle Bohemia to our cooperating clinical centre at the Third Medical Department of the General Teaching Hospital in Prague under the supervision of Václav Hána. Upon expert clinical and paraclinical evaluation by clinicians, peripheral blood samples were drawn from these probands and their families.

### 2.1.2. DNA isolation, amplification and sequencing

Genomic DNA was extracted from the anticoagulated whole blood samples in an on-site clinical laboratory by an automated magnetic-bead based DNA-extraction technique. Next, I performed an amplification of the genomic regions of interest, *i.e.* the four terminal exons of the *THRB* gene encoding the amino acid residues of the LBD of TRβ, by a polymerase chain reaction (PCR), using a high-fidelity proofreading thermostable Q5® DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The primers used were unique high-specificity oligonucleotides derived previously (Rocha et al., 2007) and listed here (Table 3).

The amplicons were purified by a column membrane purification kit Macherey-Nagel NucleoSpin® gel and PCR clean-up (Macherey-Nagel GmbH & Co. KG, Düren, NW, Germany) and sequenced in

a sequencing centre GATC Biotech AG (Cologne, Germany) by fluorescent-based capillary Sanger sequencing. All reactions were performed in duplicates to prevent polymerase-induced error and were sequenced from both sides to ensure robustness.

| | Forward | Reverse |
|---|---|---|
| **Exon 7** | 5'-GCATCTGTGTGCCTTGTCTC-3' | 5'-TGAGGTAGAAAACACTGGCATA-3' |
| **Exon 8** | 5'-CAACTTCTTCATTTAAATCTTTCTTTT-3' | 5'-ATTCCTGGAAACTGATGAAACTAT-3' |
| **Exon 9** | 5'-TGTTGTTCCTGACTGGCATT-3' | 5'-AGCGCTAGACAAGCAAAAGC-3' |
| **Exon 10** | 5'-TAAAGGCCTGGAATTGGACA-3' | 5'-GGCAATGGAATGAAATGACA-3' |

*Table 3. List of amplification and sequencing primers used in analysis of the four terminal exons of THRB gene. Reused from (Kaššák et al., 2017) based on personal use by author agreement, originally adapted from (Rocha et al., 2007).*

Feasibility analysis was performed to determine whether the same primer set can be used to amplify the genomic regions of interest and to sequence them after membrane purification. Positive results allowed a simplified protocol of genomic analysis, which could have been introduced to clinical routine after the pilot study accrual being closed. These efforts established therefore a medical screening protocol overtaken by the Institute of inborn errors of metabolism that continues to be used in clinical practice as the sole routine protocol for genomic diagnostics of RTHβ (Kaššák et al., 2017).

### 2.1.3. *In silico* structural studies and interpretation

Discovered heterozygous miss-sense mutations were subjected to a comparison in literature and databases to locate ones that were not previously described. We mapped out these changes in amino acid residues to the known crystal structures. We performed an *in silico* structural analysis of the mutated TRβs to presume impairments in its functions using a bioinformatic software tool BrowserPro (MolSoft L.L.C., San Diego, CA, USA) and evolutionary conservation analysis by the BLAST alignment tool (NCBI, Bethesda, MD, USA), presented with Jalview program (jalview.org). Finally, we compared the structural and estimated functional defects in TRβ with patient phenotype and extrapolated the functional significance of the concerned residues and determined impact on the negative regulation by thyroid hormone.

## 2.2. Experimental studies of RXR in *Trichoplax adhaerens*

### 2.2.1. Characterization of *T. adhaerens*

*Trichoplax adhaerens* was firstly discovered in 1888, only to be disregarded as a larval stage of a marine cnidarian for more than half of century. Its "rediscovery" in 1960s brought about a gradually increasing scientific interest and ever better characterisation of this missing link in the evolution of *metazoa*.

*Trichoplax* is a very flat organism typically around 500 $\mu$m to 2 mm in diameter and only about 25 $\mu$m thick. Its discoid body has a very polarized structure with distinct lower and upper (dorsal and ventral) epithelia and a middle cavity filled with water with some cells but no extracellular matrix. Currently six different cell types are known: (1) the most numerous ventral epithelial cells (VEC) responsible for feeding and movement, (2) much less abundant dorsal epithelial cells (DEC), (3) fibre cells with long dendritic processes making contacts with most remaining cells (4) gland cells producing excretion granules, (5) rare peripheral crystal cells and finally (6) more recently described lipophile cells (Figure 14) (Smith et al., 2014).

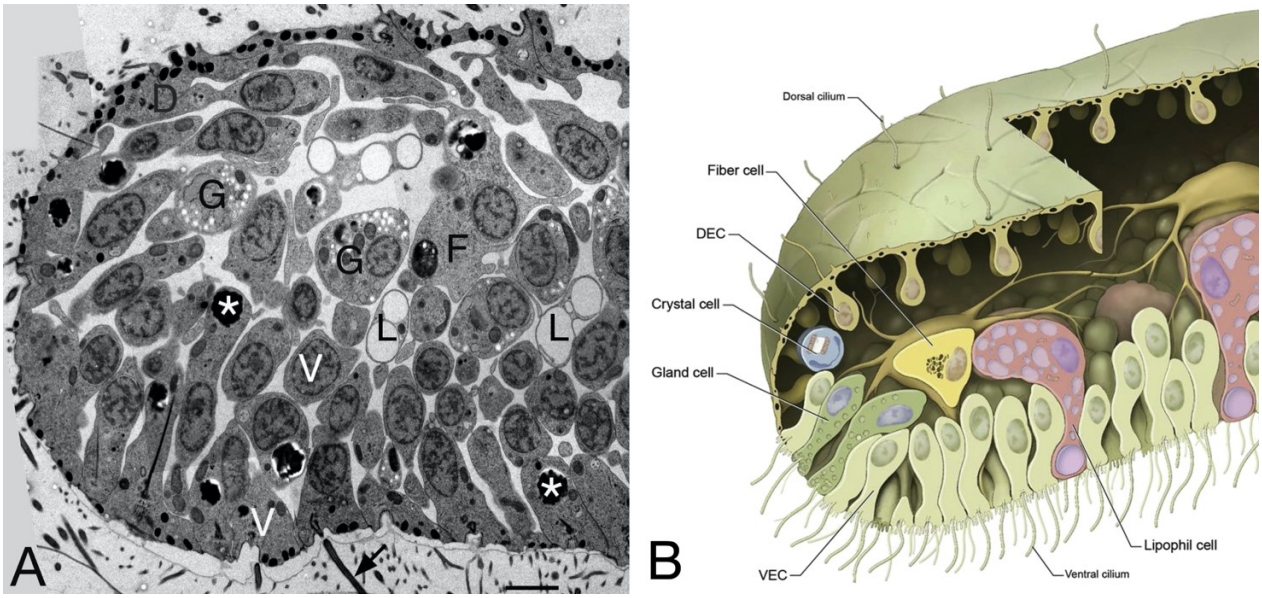


*Figure 14. Structure of T. adhaerens. (A) A montage transmission electron micrograph of a vertical section through a Trichoplax showing ventral epithelial cells (VEC, V) with their ventral cilia (arrow) and dorsal epithelial cells (DEC, D), fibre cells (F), gland cells (G) and lipophile cells (L) and dense cytoplasmic granules inside ventral epithelial cells (*). Bar in the lower right corner indicates 2 $\mu$m. (B) a schematic model of the structural organisation of a living Trichoplax indicating the cell types imaged in (A) and a supplementary crystal cell type. Adapted and modified with permission from (Smith et al., 2014).*

*Trichoplax* lives in warm coastal seas, where its nutrition consists of various unicellular and small multicellular algae. In these conditions, it needs an apparatus to sense chemical gradients in order to purposefully move in the direction of food, stalking its mobile prey. As evoked previously, NRs in simple animals could have served in communication with the environment, particularly in sensing food-associated ligands (Sladek, 2011). Accordingly, whole genome sequencing revealed four NR genes in the *T. adhaerens* genome, homologues of: ERR, HNF-4, COUP and finally RXR (Srivastava et al., 2008).

### 2.2.2. Strain and laboratory culture of *T. adhaerens*

Our culture of *Trichiplax* was obtained from the lab of Bernd Schierwater in Hannover, Germany. In laboratory conditions, the culture was kept in glass Petri dishes filled with artificial seawater prepared in our laboratory based on protocols of the Schierwater lab, based on commercial preparations (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) modified to a salinity of approximately 40 g/kg. A mixture of *Rhodomonas salina* strain CCAP 978/27, *Porphyridium cruentum* strain UTEX B637 and *Chlorella spp*. with other non-classified algae, all maintained as described previously (Kaňa et al., 2012, 2014), was used as alimentary substrate. The culture conditions were 23 °C with an artificial illumination with an automatic on-off cycle of 12 h with a natural background lightning of 2.5 h of direct and 5 h of reflected sunlight and remaining ambient dispersed lightning, from late-spring to mid-summer.

In one experiment, we analysed the influence of food composition on the phenotype of *T. adhaerens*. We constituted parallel cultures and presented different food sources to each of the cultures: *Chlorella* spp. alone, *Chlorella* spp. with *R. salina*, with *P. cruentum* or both.

### 2.2.3. RXR orthologue identification, cloning, expression and binding assays

The published *T. adhaerens* genome and predicted gene databases (Baker, 2008; Nordberg et al., 2014) were screened for the consensus DNA binding motif of NRs to locate candidate homologue genes of NRs. Apart from the previously confirmed and published ERR orthologue (Baker, 2008), candidate genes for RXR, HNF-4 and COUP-TF were found. Multispecies alignments and phylogenetic analyses were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and protein domain and module analysis was performed using Simple Modular Architecture Research (SMART) tool (Schultz et al., 1998).

*Trichoplax* total RNA was isolated using a TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed by SuperScript III RT enzyme (Invitrogen). RXR gene coding region was amplified from the cDNA using unique high-fidelity oligonucleotide primers. Amplicons were cloned into a pCR4-TOPO® plasmid vector by a TOPO TA® cloning kit (Invitrogen) and sequenced to ensure fidelity. Correct gene coding sequences were than cloned into a pGEX-2T vector adapted for bacterial expression of proteins (Addgene, Cambridge, MA, USA).

Dedicated *E. coli* strain for protein expression BL21 was transformed with the obtained pGEX-2T vector. Bacteria were grown at 37 °C until reaching optical density at 600 nm of 0.6–0.8 and then induced by 1 mM isopropyl-*D*-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) at 25 °C for 5 h. The expressed protein was purified by affinity chromatography using glutathione agarose beads (Sigma-Aldrich).

Radioactive $^{3}$H-labelled 9-*cis*-RA and $^{3}$H-labelled ATRA obtained from PerkinElmer (Waltham, MA, USA) were incubated with the produced protein, where RXR served as a stable phase in affinity chromatography while collecting wash fluid. The fraction of bound retinoic acids was determined as a ratio of bound radioactivity and total radioactivity (bound and washed out).

### 2.2.4. *In vivo* studies of RXR function in *T. adhaerens*, qPCR/ddPCR

We performed a *T. adhaerens* database search for a commonly used reporter gene of TR function, *L*-malate-NADP$^{+}$ oxidoreductase, using the Protein Basic Local Alignment Search Tool (PBLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Next, we exposed *Trichoplaxes* to a strong natural RXR ligand, 9-*cis*-RA and followed gene expression induction of the identified *L*-malate-NADP$^{+}$ oxidoreductase homologue, as well as of other genes, such as the four *T. adhaerens* identified NR genes. Animals were grown in normal conditions before being transferred to parallel cultures. After equilibration period, cultures were treated in dark with 3.3nM 9-cis-RA in 1% DMSO in ethanol vehicle or with vehicle alone.

After a 24 h incubation period, animals were counted and imaged on Olympus SZX10 microscope with DF Plan 2x objective and Olympus DP 73 camera or on CKX41 or SZX7 with Olympus E-410 camera. Thereafter, animals were collected and their total RNA was extracted using TRIZOL® reagent (Invitrogen) and reversely transcribed using SuperScript III RT enzyme and random hexamer primers (Invitrogen).

Quantitative PCR was performed independently by two separate methods: classical qPCR performed on a Roche LightCycler II with OneTaq polymerase; and highly sensitive digital droplet PCR (ddPCR) performed on a QX100 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Gene-specific oligonucleotide primers were designed, and fluorescent probes selected, using the UPL ProbeFinder software (Roche, Basel, Switzerland). Absolute transcript copy number acquired by ddPCR was analysed using the Bio-Rad ddPCR software. Relative expression change was deducted by subtracting control.

## 2.3. Identification of MED28 and Perilipin orthologues in *C. elegans*

### 2.3.1. Introduction to *C. elegans* biology, methodology and lipid metabolism

Currently being used as one of two predominant invertebrate animal model organisms, *C. elegans* was first isolated as a ubiquitous nematode found in decomposing vegetable matter, 120 years ago (Maupas, 1900). Currently, this animal model is utilized by over 1 000 specialised laboratories in the world, including ours. Among the immense benefits offered by this model system is its short life cycle, rapid reproduction rate, simple and discernible anatomy thanks to its transparency, relatively small genome with short introns, as well as an extensive repertoire of established technologies, most notably RNA interference (RNAi) and CRISPR/Cas9 endonuclease. At the same time, in comparison to other model mechanisms, *e.g., D. melanogaster*, nematodes diverged less from a last common ancestor with human, hence many genes and pathways are better conserved and discoveries have better transferability.

*C. elegans* adult body is formed by a constant number of about 1 000 somatic cells and reaches up to 1 mm in size. The hermaphrodite anatomy is built around a longitudinal gut lumen lined by enterocytes, with large paired kinked gonads meeting in uterus, all surrounded by a pseudocelomic cavity and a body wall with muscles, neurons, seam cells and hypodermis secreting cuticle. There are two sexes: hermaphrodites with genotype XX and males with genotype XO. Hermaphrodites are self-fertilising their eggs in pair organs called spermathecas. Males serve solely to increase recombination rate in the population and are relatively rare (Altun & Hall, 2009).

In laboratory conditions, *C. elegans* cultures are grown in Petri dishes on pepton-enriched agar plates seeded with *E. coli* strain OP50. Their life cycle is strongly influenced by temperature. At 22 °C, laid embryos take about 9 h to hatch into first larval stage (L1). Larval development continues through

stages L2 through L4 separated by molt events, taking in total approximately 36 h until young adult stage. In another 10 h, adults start laying embryos (Figure 15). This whole cycle takes around 55-60 h at 22 °C, but only 40 h at 25 °C and up to 90 h at 16 °C. The total lifespan at 22 °C is about 2-3 weeks. After the L1 stage, in unfavourable conditions, especially in scarcity of food, a special intermediate phenotype called *dauer* larva can be formed. *Dauers* can survive up to six months without food, always retaining the possibility to escape the *dauer* stage to L4 stage upon food reappearance (Altun & Hall, 2009).

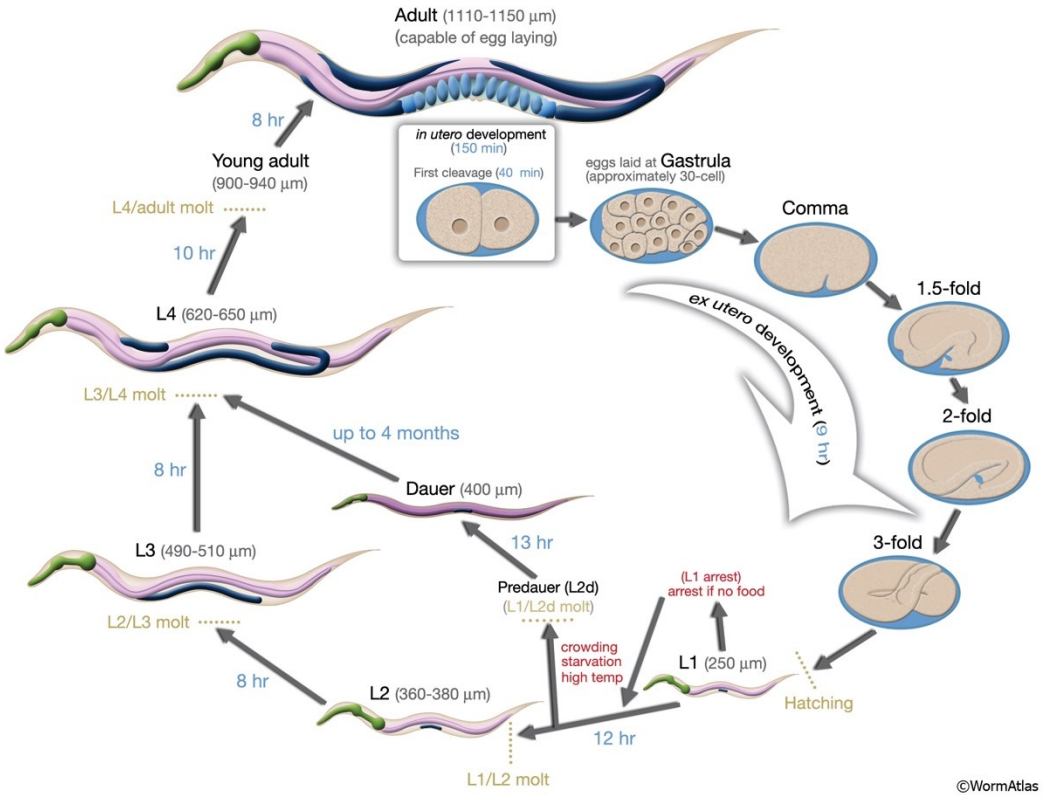


*Figure 15. C. elegans growth cycle. Reused with permission from (Altun & Hall, 2009).*

As virtually all animals, *C. elegans* utilize tri-acyl glycerol (TAGs) for excess energy storage. These are localized to cytoplasmic organelles called lipid droplets (LDs). Where once LDs were considered passive drops of fat surrounded by a monolayer of amphipathic lipids, we now know that in all studied eukaryotic organisms LDs are extremely dynamic and closely regulated structures. LDs are present in most cells, but some cells are specialized in lipid storage and their LDs are much more prominent, *e.g.,* white and brown adipocytes in mammals and enterocytes in *C. elegans* (Watts & Ristow, 2017).

When free energy liberation is required, TAGs are broken down into di-acyl and mono-acyl glycerol (DAGs and MAGs) and free glycerol, each time removing one non-esterified fatty acid (NEFA) that

is addressed to β-oxidation. This hydrolysis of glycerol-ester bonds in TAGs is the rate-limiting step in lipolysis and as such is precisely regulated. Two pathways catalyse the lipid breakdown: (1) conventional cytoplasmic lipolysis by neutral lipases; and (2) lipophagy, a lipid directed type of autophagy, by acidic lipases inside autophagosomes). Neutral lipases catalyse lipolysis in two steps: adipocyte triglyceride lipase (ATGL, nematode orthologue ATGL-1) catalyses TAG to DAG hydrolysis and hormone sensitive lipase (HSL, nematode orthologue HOSL-1) catalyses DAG to MAG and to free glycerol lysis (Granneman et al., 2007).

These lipases are controlled by the central regulators of lipolysis, LD surface-resident proteins Perilipins (PLINs) (Kimmel et al., 2010). In human, five PLIN paralogues have been identified to date. All of these have conserved structure, but differing, even opposing functions and differential tissue expression profile (Itabe et al., 2017). PLIN orthologues have been identified in many distant phyla of the eukaryotic realm, including fungi and amoebas, but no PLIN orthologue had been known in *C. elegans* (Bickel et al., 2009).

### 2.3.2. Identification of MED28 and PLIN orthologues

Considering the seemingly unrelated function of the nematode *MED28* homologue gene of the locus W01A8.1 called *mdt-28*, we decided to revisit the annotation of this gene as MDT-28.

A multi-platform search of the *MED28* sequence and of the W01A8.1 locus was performed in the NCBI (ncbi.nlm.nih.gov), the UniProtKB (uniprot.org) and the OMA databases (omabrowser.org) by BLAST, PSI-BLAST (Altschul et al., 1997), HHblits (Remmert et al., 2012) and HHpred (Söding et al., 2005) applications, while reiterating the results by secondary structure prediction via PSIPRED (Jones, 1999). A T-Coffee algorithm alignment (Notredame et al., 2000) of derived sequences was executed and the alignments were resubmitted to PSI-BLAST with an E-value cut-off of $10^{-3}$ upon 5 iterations. Sequences of the two newly distinguished genes were displayed with their respective PLIN and MED28 homologues using Jalview application (jalview.org).

### 2.3.3. Protein binding studies

Having identified an alternate locus F28F8.5 as the likeliest candidate orthologue gene for a MED28 orthologue, its homology was ascertained by binding studies with the Mediator subunits known to

bind MED28 in human: MED6 and MED23. The *F28F8.5*, *mdt-6* and *mdt-23* coding sequences were amplified by PCR from total *C. elegans* cDNA (prepared as described below) by PCR

The *F28F8.5* amplicon was cloned into pGEX-2T vector (Amersham Pharmacia Biotech, Amsterdam, UK) using the GeneArt Seamless Plus Cloning and Assembly Kit (Thermo Fisher, Waltham, MA, USA) and transformed into BL21 *E. coli* strain. The *mdt-23* amplicon was cloned into the pET28a(+) vector (Addgene) containing sequences for $His_6$ and FLAG tags, using Quick Ligation Kit (New England Biolabs) and transformed also into BL21 bacterial cells. Protein expression of both GST::F28F8.5 and of $His_6$::MDT-23::FLAG in the bacteria was induced by IPTG.

The amplicon of the *mdt-6* gene was cloned into a pTNT vector (Promega, Madison, WI, USA) using the Quick Ligation Kit (New England Biolabs). The MDT-6 protein was produced by *in vitro* transcription and translation in the rabbit reticulocyte lysate TNT-system (Promega) with inclusion radioactive $^{35}S$ Methionine (Institute of Isotopes, Budapest, Hungary).

The binding studies were performed on glutathione agarose (Sigma-Aldrich) saturated with either GST alone or GST::F28F8.5. After incubation with either $^{35}S$-Met-MDT-6 or with $His_6$::MDT-23::FLAG, the labelled proteins bound to either GST or GST:: F28F8.5 were separated using native polyacrylamide gel electrophoresis (PAGE). Presence of $^{35}S$-Met-MDT-6 was then established using autoradiography and presence of $His_6$::MDT-23::FLAG was evidenced by monoclonal M2 anti-FLAG antibody (Sigma-Aldrich) Western Blotting.

## 2.3.4. Strains and transgene preparation

We utilize the N2 var. Bristol *C. elegans* strain as the standard wildtype line (Brenner, 1974). In the efforts leading to the characterisation of W01A8.1 as the PLIN homologue, I also employed lines VS20 [$P_{atgl-1}$-*atgl-1::gfp* + *mec-7::rfp*] obtained from the Caenorhabditis Genetic Center of the University of Minnesota (Minneapolis, MN, USA), tm2369 [*hosl-1*$^{-/-}$] from the National BioResource Project (NBRP) (shigen.nig.ac.jp/c.elegans/top.xhtml) and RD204 [$P_{pie-1}$-*lgg-1::gfp*] gifted from the laboratory of Dr Renaud Legouis (Manil-Ségalen et al., 2014).

In an effort to identify the W01A8.1 protein as a PLIN homologue rather than that of MED28, we prepared plasmid vectors containing nematode W01A8.1 isoforms and human PLIN paralogues with in-frame C-terminal enhanced green fluorescent protein (eGFP). Shortly, W01A8.1 isoforms (b) and (a/c) were cloned from isolated *C. Elegans* DNA and the isoform (a) with same-sense polymorphisms

protecting it from endonuclease cleavage was purchased as a nematode-adapted synthetic gene string from Invitrogen. Human paralogues *PLIN2* and *PLIN3* were cloned from donated peripheral blood sample upon ethics committee approval (Ref. MZ13-UK1LF-KostrouchZdenek) and from unmarked samples. Human *PLIN1* sequence was obtained as nematode-adapted synthetic sequence string from Invitrogen. All nematode and human isoforms were cloned into vectors containing in-frame eGFP in the C-terminal position.

### 2.3.5. Genome editing using CRISPR/Cas9 enzyme

In order to produce targeted knock-out and knock-in phenotypes in the studied genes, we employed a modern genome editing technique based on the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system. This system originates in prokaryotic and archaea cells, where it functions as a mechanism of immune defence against bacteriophages (Heler et al., 2015). It has been in the spotlight of recent biological research thanks to its applications in genome editing. Cas9 is an extremely specific endonuclease that utilizes three distinct RNA molecules to target a dsDNA sequence, inducing a double-strand break through its two nuclease domains (Jinek et al., 2012). Thanks to this function, it has been rapidly adapted to utilization for gene deletion and homologous recombination in a great variety of organism, including *C. elegans* (Friedland et al., 2013).

The predominant currently used nematode CRISPR/Cas9 system uses a single plasmid vector denoted pJW1219 encoding both the Cas9 enzyme and a sequence of fused sgRNA (Dickinson et al., 2013). For purposes of induced homologous recombination with insertion of an N-terminal fluorescent protein sequence, sophisticated selection vectors have been prepared, which contain fluorescent protein and a LoxP site-flanked Self-Excision Casette (SEC). This SEC contains double selection of *sqt-1(d)* transcript causing Rol phenotype and hygromycin resistance gene, as well as the Cre recombinase gene under a heat-shock promoter cutting the LoxP sites around the SEC itself upon heat-shock (Dickinson et al., 2013). Both pJW1219 and pDD122 (GFP-SEC vector) were purchased from AddGene (AddGene, Cambridge, MA, USA).

We identified two separate Cas9 targets inside the W01A8.1 gene sequence and cloned these sequences into two separated vectors using PCR-based site-directed mutagenesis of the pJW1219 vector. In the same way, we prepared a vector containing sgRNA targeted close to the beginning ATG of the F28F8.5 locus (identified as possible MED28 homologue). We also prepared a recombination construct by cloning 5'- and 3'-flanking regions of the *F28F8.5* gene beginning upstream and

downstream of the GFP-SEC sequence of the pDD122 vector. For this we amplified the homology regions from isolated nematode gDNA using primers with flanking sequences complementary to those of the pDD122 vector and cloned them using Gibson Assembly cloning kit (New England BioLabs, Ipswich, MA, USA) (Figure 16).

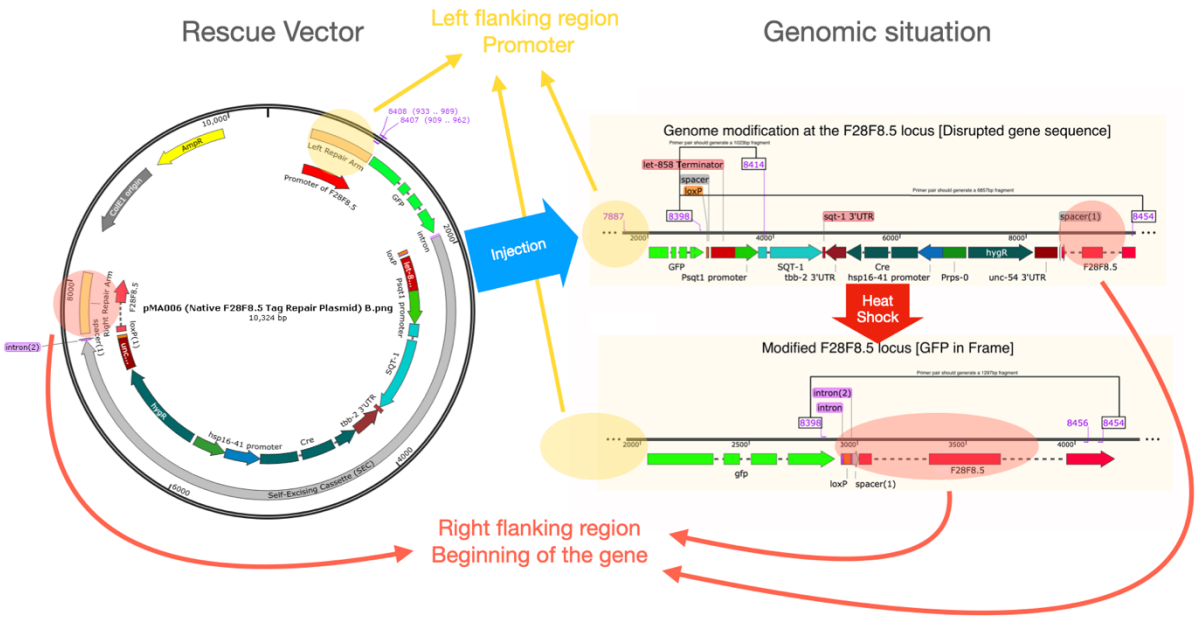


*Figure 16. Diagram of cloning and recombination events leading to genomic incorporation of GFP-SEC at the N-terminus of the F28F8.5 gene. Figure modified from (Kostrouchová et al., 2017) originally published under the Creative Commons Attribution Licence.*

## 2.3.6. Injections, transgene screening, swPCR, genetic crossing

The above-described vector constructs were next mixed with co-injection markers pGH8, pCJF104 and pCJF90 expressing a red fluorescent protein mCherry transcriptionally fused with three distinct unrelated proteins, to allow for a more robust screening of positive progeny. The injection mixes were injected into gonads of young adult hermaphrodites using Olympus IX70 microscope equipped with a Narishige microinjection system (Olympus, Tokyo, Japan) as described previously (Tabara et al., 1999).

Injected hermaphrodites were placed on individual plates and their $F_1$ progeny was screened for red co-injection markers using a fluorescent dissection microscope. SEC-containing constructs were additionally subjected to Hygromycin plate selection and plates were screened for Rol phenotype and

rolling surviving worms were heat-shocked at 34 °C for 4h. The presence of intended genotype was then verified by specific single-animal PCR (swPCR) and sequencing. Single positively screened hermaphrodites were transferred to individual plates, let to lay progeny, then lysed by Proteinase K in Barstead buffer. The lysate was diluted and directly used as template for gene-specific PCR amplification and the corresponding size of the applicates was verified by agarose gel electrophoresis. Positive lines were finally harvested, their DNA isolated, a greater genomic segment amplified and sequenced using modified Sanger sequencing as described above by GATC Biotech AG (Cologne, Germany).

Having produced our W01A8.1$^{-/-}$ null mutant denoted KV1, we performed genetic crosses of this line with the externally obtained lines RD204, VS20 and tm2369 to produce respectively: KV2 [$P_{pie-1}$-*lgg-1::gfp* + *W01A8.1*$^{-/-}$], KV7 [$P_{atgl-1}$-*atgl-1::gfp* + *W01A8.1*$^{-/-}$ + *mec-7::rfp*] and KV10 [*hosl-1*$^{-/-}$ + *W01A8.1*$^{-/-}$] lines. Successful crosses were screened using swPCR and sequenced as described above.

### 2.3.7. RNA interference

One of the reasons for the success of the *C. elegans* as a model system was an early adaptation of the RNA interference (RNAi) methodology, which awarded Andy Fire and Craig Mello a Nobel prize in medicine and physiology in 2006. This simple gene silencing method exploits native short interfering RNAs (siRNAs) naturally implicated in viral immunity. Double-stranded RNA (dsRNA) binds to a protein called Dicer that cleaves and denatures the dsRNA. Single-strand RNA (ssRNA) fragments are then mounted into an RNA-Induced Silencing Complex (RISC). In this complex, ssRNA acts as a template which guides the RISC to mRNA that are thereby cleaved (Grishok, 2005).

In an experimental setting, dsRNA templates of genes to be downregulated are transported inside nematodes by injections, soaking or feeding(Ahringer, 2005). We employed injections and feeding protocols in our experiments. Fragments partially covering the target gene coding sequence were amplified form total nematode cDNA prepared by reverse transcription using SuperScript III RT (Invitrogen) with oligo-T primers from total RNA isolated by TRIZOL® reagent (Invitrogen). These fragments were then cloned into pCRII vector (Thermo Fischer) or L4440 vector (AddGene) for application in injection and feeding respectively.

For injection, dsRNA was prepared by *in vitro* transcription using SP6/T7 Riboprobe systems® (Promega) from linearized plasmid. The dsRNA was injected into young adult hermaphrodite germline as described above. For feeding, HT115 strain of *E. coli* was transformed with the L4440

plasmid containing target sequence. The dsRNA transcription in these bacteria was induced by IPTG inside prepared agar plates, on which nematodes were grown. The efficacy of RNAi was verified by qPCR techniques using LightCycler real-time fluorescent thermal cycler (Roche, Basel, Switzerland) with gene-specific primers and universal probes.

### 2.3.8. Cold exposure experiment

To show functional connection of W01A8.1 and the crucial lipase HOSL-1, we employed an experimental setup specifically dissecting the function of HOSL-1 based on cold exposure tolerance. Animals exposed to temperatures close to 0 °C trigger PKA-dependent HOSL-1-mediated lipolysis that produces cryoprotective glycerol (Zhu et al., 2017).

I exposed wild-type controls, *W01A8.1* mutants, *hosl-1* mutants and *W01A8.1 hosl-1* double mutants to temperatures of 0-0.5 °C for 40 hours. Afterwards, I scored their survival and imaged surviving animals stained by LipidTox to ascertain association of survival with lipolysis (Kaššák et al., 2020).

### 2.3.9. Fluorescent, confocal, FLIM and CARS microscopy

We employed a wide array of modern microscopic techniques in our direct studies of *C. elegans*. Classical fluorescent images were acquired alongside differential interference contrast (Nomarski optics) images using Olympus BX60 microscope equipped with excitation and emission filters of red and green spectrum fluorescence maxima and with a DP30BW CD camera. Animals were captured mounted on a 2% agarose-covered glass slides and immobilized by levamisole solution.

Confocal images were captured either on the inverted Leica SP8 TCS SMD FLIM system equipped with a 63 x 1.2 NA water immersion objective, a pulsed white light laser (470–670 nm), AOBS and two internal hybrid single photon counting detectors (Leica Microsystems, Wetzlar, Germany), or on the Carl Zeiss LSM 880 NLO microscope with the Chameleon Ultra II and Chameleon Compact OPO MP lasers (Coherent, Santa Clara, CA, USA) and a LD C-Apochromat 40x/1.1 W Corr M27 objective (Carl Zeiss AG, Oberkochen, Germany). Four-dimensional hyperstacks of *z*-stacks of *xy* planar images with continuous emission spectroscopy were captured.

Fluorescence Lifetime Imaging Microscopy was employed in order to distinguish autofluorescence from proper GFP signal. Leica SP8 TCS SMD FLIM system was also used for these experiments.

Four-dimensional *xyz* images with fluorescent decays time spectrum were captured in TTTR data format and processed by HydraHarp400 TCSPC electronics (PicoQuant, Berlin, Germany).

In our lipid metabolism studies, Coherent Anti-Stokes Raman Spectrocopy (CARS) was used. This technique is based on characteristic vibration spectra of individual molecular bonds, in this case of the $CH_2$ bonds abundantly present in neutral lipids. The sample is simultaneously illuminated with two laser beams at different frequencies (pump/probe and Stokes beam), separated by a vibrational frequency of the target molecule. In a spatial context, this allows excellent biological imaging of molecules rich in the given pattern (Hellerer et al., 2007; Schindelin et al., 2012). We used Leica TCS SP8 CARS system (Leica Microsystems, Mannheim, Germany) consisting of a TCS SP8 confocal microscope combined with a picoEmerald (APE, Berlin, Germany) fixed Stokes laser line of 1064.5 nm and a tuneable Pump line set to 815.5 nm for imaging of $CH_2$ with a Raman shift of 2,868 $cm^{-1}$. Alternatively, where a concurrent confocal image was required, the aforementioned Carl Zeiss LSM 880 NLO microscope with a modification permitting simultaneous pump and Stokes beam excitation was employed.

All images were analysed using ImageJ program under the distribution Fuji (Schindelin et al., 2012). Representative pictures alongside morphometric analyses are presented.

# 3. Results

## 3.1. Negative regulation by TRs depends on coactivator binding by TRβ in a mutation model provided by RTHβ

Our clinical accrual found 17 suspected cases of RTHβ with the distinct biochemical phenotype of elevated $T_3$ and $T_4$ levels and non-suppressed TSH levels. In total, there were four families of 4, 4, 2 and 2 patients and three single probands without possibility of familial analysis. Genomic analysis revealed three previously described and studied mutations and one previously unknown mutation in the four terminal exons of the *THRB* gene (Kaššák et al., 2017).

In one family of four a heterozygous adenine to guanine mutation (A/G) at the position 1327 of the TRβ1 coding sequence (CDS) was found. This mutation translates to a miss-sense change of lysine to glutamic acid in the position 443 (K443E) and has been described previously (Sasaki et al., 1992). Another family of two harboured a heterozygous T/C mutation in the nucleotide 1375 resulting in F459L (C. S. Mitchell et al., 2010). Another single proband had an A/G mutation at position 962 of CDS leading to Y321C change (Adams et al., 1994).

In one family where the proband mother and her daughter were analysed, *THRB* sequencing found a heterozygous miss-sense mutation changing a single cytosine to guanine at the position 818 of the TRβ1 CDS. Resulting amino acid change of threonine to arginine at the position 273 (T273R) has not been previously described in scientific literature. Therefore, we decided to describe the phenotype in detail and to analyse *in silico* the impact of this mutation on the function of TRβ, concentrating on the negative regulation exerted by this NR.

At the time of diagnosis, the proband with RTHβ based on T273R mutation in *THRB* was a 37-year-old woman exhibiting mixed symptoms of developmental disorders, hypothyroidism, hyperthyroidism and the characteristic biochemical phenotype. Specifically, she suffered from severe attention deficit/hyperactivity disorder (ADHD) leading to educational, professional and secondary psychological difficulties. At the same time, she presented increased transpiration and heightened tolerance to cold. Paraclinical investigations revealed elevated serum $fT_4$ levels at 29.38 pM, *i.e.* almost triple the upper normal limit, with non-supressed serum TSH levels at 2.21 mIU/l, indicating defective negative feedback regulation. No other symptoms associated with RTH, such as tachycardia, goitre or hearing impairment were present.

Proband's daughter of four years old had experienced discreet failure to thrive, initially retarded development and a diffuse goitre of 6.6ml at 33 months. Biochemical investigation revealed even higher fT4 levels (41.97pM) than in her mother and even slightly elevated TSH levels (4.97mIU/l).

The origin of the mutation seems familial, passed down from the proband's father, although no genetical confirmation could have been accomplished in regard to the father's demise several years before the initiation of our study. The father had been known for endocrinological disturbances and a comparable biochemical phenotype. Proband's living mother had had no thyroid axis problems.

Next, the mutation was analysed from an evolutionary perspective. Sequence alignments with representative species of the whole evolutionary branch of species with known TR orthologues were prepared. The threonine in the position 273 of TRβ1 CDS is completely conserved throughout the subphylum of vertebrates and in most chordates and is only divergent in the subphylum *cephalochordata*, as demonstrated in the lancelet *Brachistoma floridae* (Figure 17).

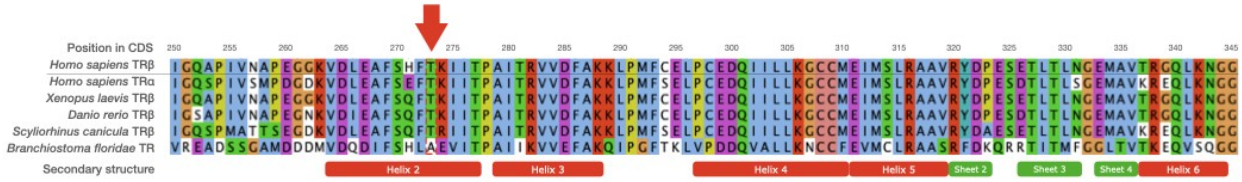


*Figure 17. Evolutionary conservation of the primary structure of TRβ LBD cantered around the newly discovered mutation. Red arrow points to the locus of the mutation T273R. Secondary structure is represented below the alignment. Figure adapted and expanded, based on the one previously published (Kaššák et al., 2017) with personal reuse by author agreement.*

Finally, an *in silico* analysis was performed. The novel mutation T273R was mapped to the helix 2, with its residue not contributing to the ligand binding pouch but rather tightly packing against the loop connecting helices 11 and 12 (Figure 18). Indeed, applying secondary structure algorithms JPred and PSIPRED (Jones, 1999) based on biochemical interactions reiterated by a large database of homologous protein structures, we predicted with a high degree of certainty that an arginine in the location 273 does not impair $T_3$-binding, but its bulkier charged residue inhibits the mobility of the helix 12, effectively interfering with its clamp function in the AF2 subdomain. As explained in section 1.2.2., the mobility of helix 12 and an undisturbed AF2 surface are crucial for coactivator binding.

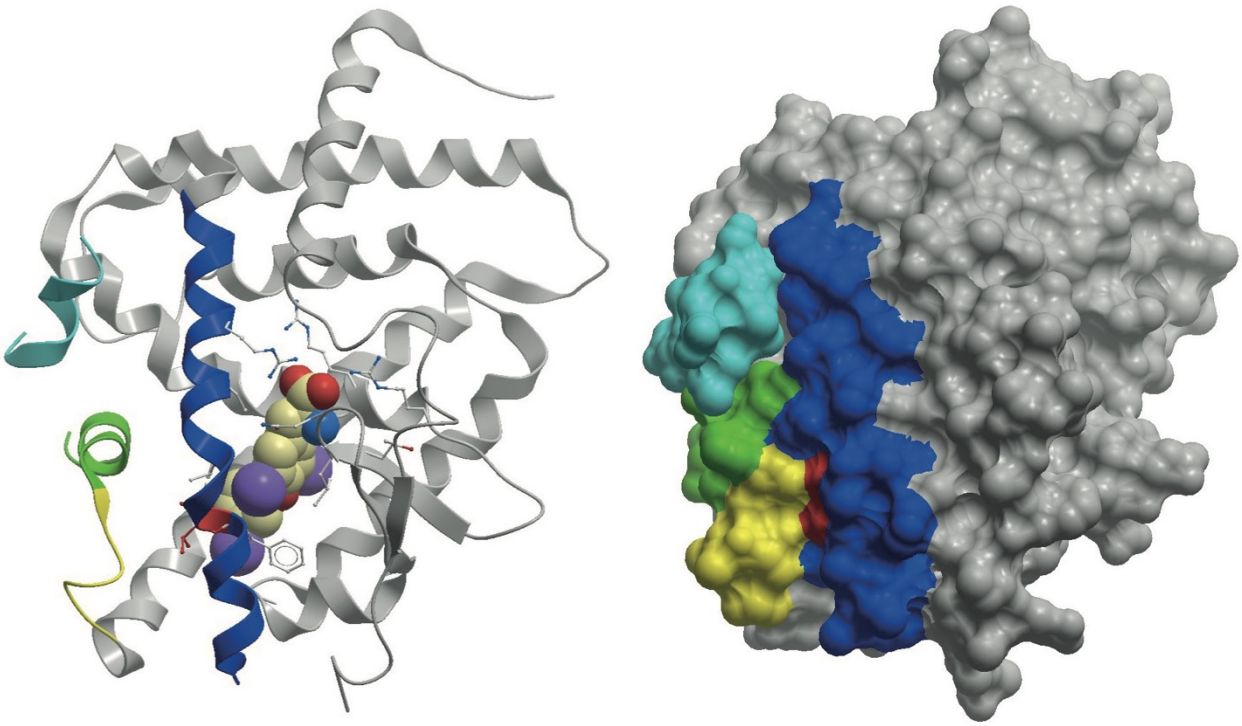

*Figure 18. Secondary structure of the TRβ LBD binding $T_3$ (space filling model) and a coactivator consensus motif LxxLL (cyan, not a part of TRβ molecule). Position of the mutation T273R (red) within the helix 2 (dark blue) suggests direct interaction with the loop connecting helices 11 and 12 (yellow) and the helix 12 itself (green). Reused from (Kaššák et al., 2017) based on personal reuse by author agreement.*

## 3.2. *T. adhaerens* possesses a functional RXR orthologue implicated in alimentary sensing

In the second part of this work, my colleagues and I concentrate on TR binding partner Retinoid X Receptor (RXR) that is evolutionarily more ancient, and we postulate that it had been responsible for metabolic and developmental regulation before TRs took over partially this role in chordates. We selected *Trichoplax adhaerens* for RXR studies due to its placement in the evolutionary tree with little divergence from common ancestor with human and sufficiently distant for evolutionary studies.

First, we identified the likely *bona fide* RXR orthologue in *T. adhaerens* via a JGI database search and high-throughput sequence alignments. A previously not annotated sequence was identified as the best candidate for an RXR homologue gene. Sequence analysis revealed a striking 66% overall identity to human RXRα with the most critical domains being completely similar, *e.g.,* the DBD and the LBD were similar with E values < $10^{-40}$. Within the LBD, an almost complete identity and a complete similarity was present among the 11 residues critical for ligand-binding and complete identity was present in the dimerization subdomain (Figure 19).

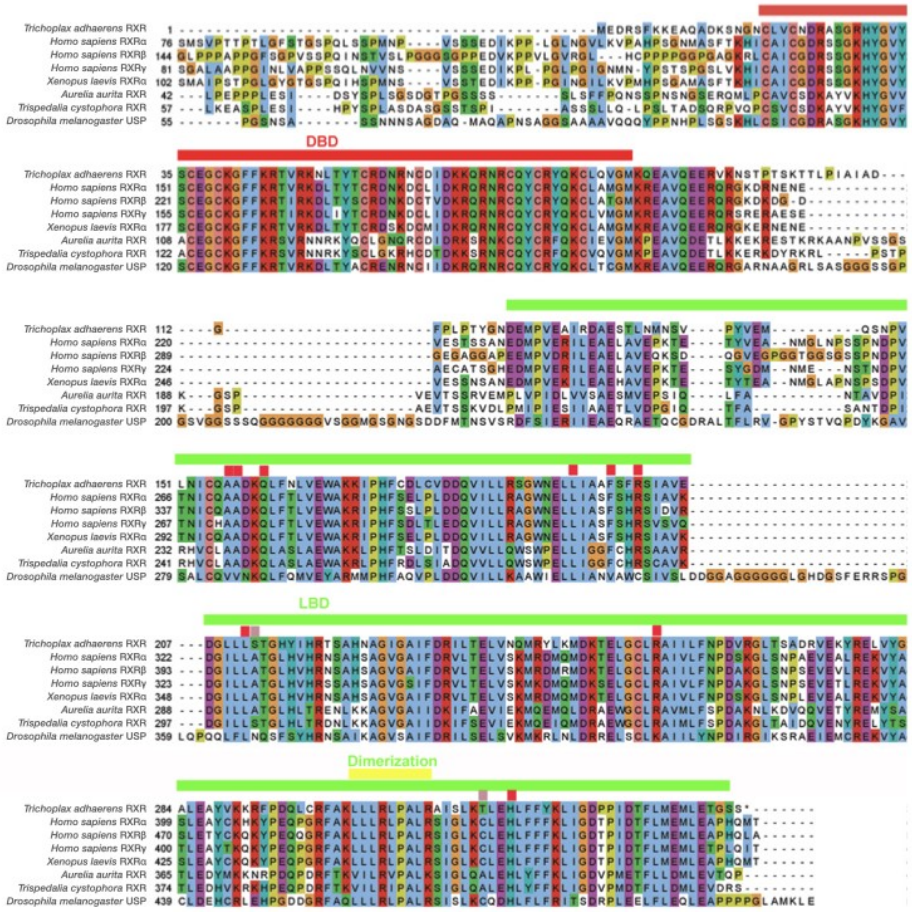


*Figure 19. Sequence alignment of T. adhaerens RXR with RXR orthologues of various species. DNA binding domain (DBD, red line), Ligand binding domain (LBD, green line), dimerization domain (yellow line) and amino acid residues critical for 9-cis-RA binding (conserved—red rectangles, not conserved—pink rectangles) are indicated. Figure modified from (Novotný et al., 2017) originally published under the Creative Commons Attribution Licence.*

To verify the preservation of ligand-binding and of gene expression regulation functions, we performed am *in vitro* ligand binding. We incubated $^{3}$H-labelled 9-*cis*-RA and $^{3}$H-labelled ATRA with bacteria-expressed TaRXR and determined binding by measuring total bound radioactivity and the radioactivity displaceable by 200-fold excess of nonradioactive competitors. We showed that TaRXR binds 9-*cis*-RA with a higher affinity than ATRA with a binding plateau at 3 to 5 nM (Figure 20).

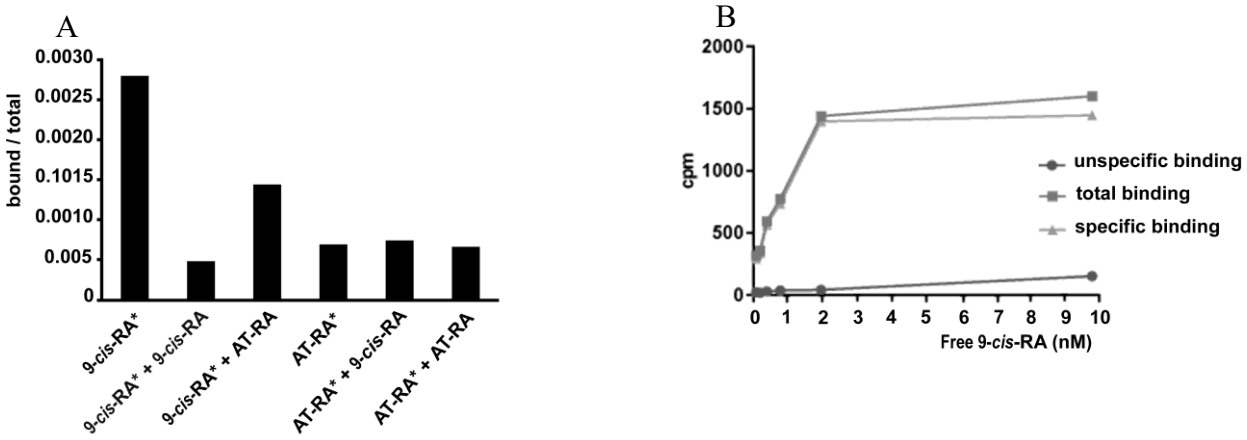


*Figure 20. In vitro binding studies. (A) Point analysis of binding of $^{3}$H-9-cis-RA at a concentration of 4 nM per 200 ng of RXR defined as ration of bound to total radioactivity. 200-fold excess of non-radioactive 9-cis-RA displaces most radioactive isotope, whereas a 200-fold excess of ATRA only displaces about 50% of the bound radioactive 9-cis-RA. Radioactive $^{3}$H-ATRA only binds to RXR with an affinity that is non-specific. Radioactive isotopes marked with as asterisk. (B) Kinetic binding analysis of $^{3}$H-9-cis-RA at increasing concentrations shows a binding plateau at 3 nM to 5 nM concentration. Figure modified from (Novotný et al., 2017) originally published under the Creative Commons Attribution Licence.*

Next, we analysed the function of TaRXR *in vivo* by exposing live *Trichoplaxes* to 9-*cis*-RA, while following the expression of their supposed target genes, specifically the *L*-malate-NADP$^{+}$-oxidoreductase homologue and of the four identified NR gene homologues. Quantitative digital droplet PCR showed a highest 4-fold increase in the malic enzyme mRNA levels after treatment with 3 nM 9-*cis*-RA, a milder 2-fold increase upon treatment with 30 nM 9-*cis*-RA and no effect of 30 nM ATRA treatment on the malic enzyme expression (Figure 21A).

Expression changes of the four NR homologues upon 9-*cis*-RA treatment were also measured. The effect is highly pronounced in lower rather than higher concentrations of the ligand. 9-*cis*-RA-induced RXR activation increases the relative expression of RXR itself and of ERR, has no effect on HNF4 orthologue expression, but strongly downregulates the expression of COUP-TF homologue (Figure 21B).

Finally, we assessed the role of RXR signalling in *T. adhaerens* by showing interaction of the pathway with feeding conditions. First, we established the influence of food composition on *T. adhaerens* proliferation. We grew parallel cultures of an identic number of animals with different composition of algal food. In the control experiment, *Trichoplaxes* were fed green algae of the *Chlorella* spp. prepared by long-term aquarium culture and standardly used in culture maintenance. Animals kept in this way doubled their number in the 8-day observation period.

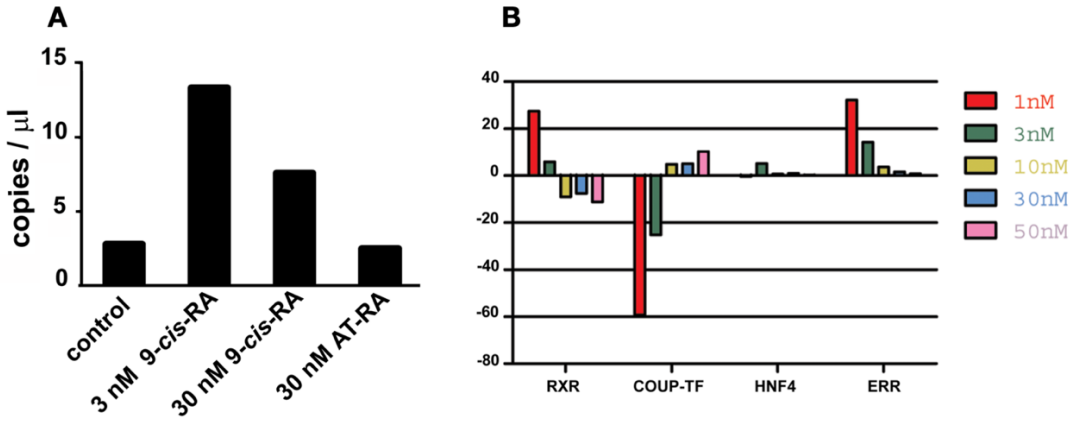


*Figure 21. Functional studies of RXR ligands in vivo. (A) Gene expression upregulation of L-malate-NADP$^+$-oxidoreductase enzyme in T. adhaerens upon treatment with different concentrations of 9-cis-RA and with ATRA defined as absolute mRNA copy number per µl. (B) Changes in expression of the four NR homologues relative to the reminder upon treatment with various concentrations of 9-cis-RA defined as percent change. Figure modified from (Novotný et al., 2017) originally published under the Creative Commons Attribution Licence.*

However, when red algae *Rhodomonas salina* and *Porphyridium cruentum* had been progressively added to the culture, the proliferation rate was strongly increased. Animals multiplied 5-times (*Chlorella* spp. and *R. salina*) or even 10 times (*Chlorella* spp., *R. salina* and *P. cruentum*) in number (Figure 22A). This effect was therefore additive in between individual red algal species but was not replicated by increasing the amount of available food, only by its composition.

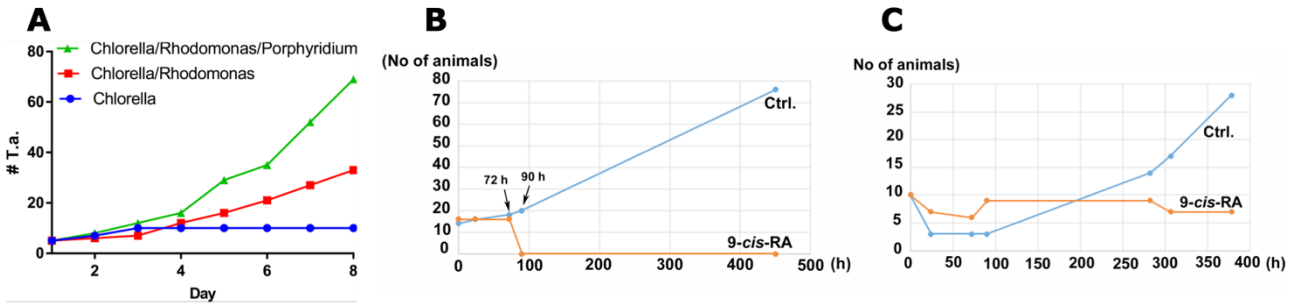


*Figure 22. Effect of differential food composition and 9-cis-RA on T. adhaerens culture. (A) Culture proliferation as a function of differential food composition shows stark increase in proliferation upon introduction of red algae Rhodomonas salina and Porphyridium cruentum. (B) Effect of exposure to 3.3 nM 9-cis-RA on a culture fed on P. cruentum shows a delayed decay and death phenotype. (C) Exposure to 9-cis-RA in a control culture fed on Chlorella spp. shows proliferation arrest but not extinction of treated animals. Figure modified from (Novotný et al., 2017) originally published under the Creative Commons Attribution Licence.*

Next, we evaluated the interconnection of feeding and RXR signalling. In cultures fed by standard aquarium *milieu* with *Chlorella* spp., treatment with 3.3 nM 9-*cis*-RA led to proliferation arrest persisting for at least 16 days (Figure 22C). In cultures where animals were fed the red algal species *P. cruentum*, the effect of the 9-*cis*-RA exposure was much stronger with formation of a typical balloon-like phenotype and death of all studied animals by day 4 (Figure 22B).

## 3.3. Identification of MED28 and PLIN orthologues in *C. elegans* supports conservation of Mediator and of perilipin-based regulation of lipolysis in nematodes

In *Caenorhabditis elegans*, most Mediator subunits are conserved to a degree that allows their identification by widely used bioinformatics tools. One exception seems to be the subunit 28, the nematode homologue of which had been identified as a gene in the locus W01A8.1. However, there is little homology between mammalian MED28 and *C. elegans* W01A8.1, suggesting possible incorrect annotation of W01A8.1 as *mdt-28*.

Similarly, it was thought that *C. elegans* lipolysis was not regulated by perilipin proteins (PLINs), as no PLIN orthologue had been found. This motivated a bioinformatical sequence analysis performed by Vladimir Saudek, which suggested a significant degree of homology between W01A8.1 and PLINs in a variety of organisms. To confirm this homology bio-informatically, he derived a Hidden Markov profile from a widespread sequence analysis by HHrepID algorithm. In this way, the predicted homology of W01A8.1 and human PLINs was confirmed with a high degree of confidence (down to $E = 10^{-53}$). An inverse direct homology alignment of the human PLIN2 and PLIN3 paralogues also identified the W01A8.1 protein ($E = 3 \times 10^{-13}$). Structural analysis recognized the three PLIN definition sequences in the *W01A8.1* gene: N-terminal Patatin-like domain (PAT), an imperfect amphiphilic 11-mer domain and a C-terminal four-helix bundle (Figure 23B).

Successively, in order to strengthen his prediction, he searched for a possible MED28 homologue. An *ab initio* database search of several MED28 sequences from organisms throughout the phylogenetic tree identified a different candidate gene as the closest likely MED28 orthologue. The gene annotated as *F28F8.5* was identified as homologous to human MED28 in iterative alignments with high specificity (significance score down to $E < 10^{-48}$) and reciprocal alignments ($E < 10^{-8}$). Probability of F28F8.5 being a *bona fide* MED28 was calculated at >99.99% (Figure 23A). In contrast, he found no significant homology between the MED28 homologues throughout the animal kingdom and *W01A8.1*. The annotation of *W01A8.1* as *mdt-28* was traced back to a Hidden Markov

model seed homology between bovine and mosquito MED28 in the Pfam database (Punta et al., 2012), from where all subsequent automatic annotations were incorrectly derived.

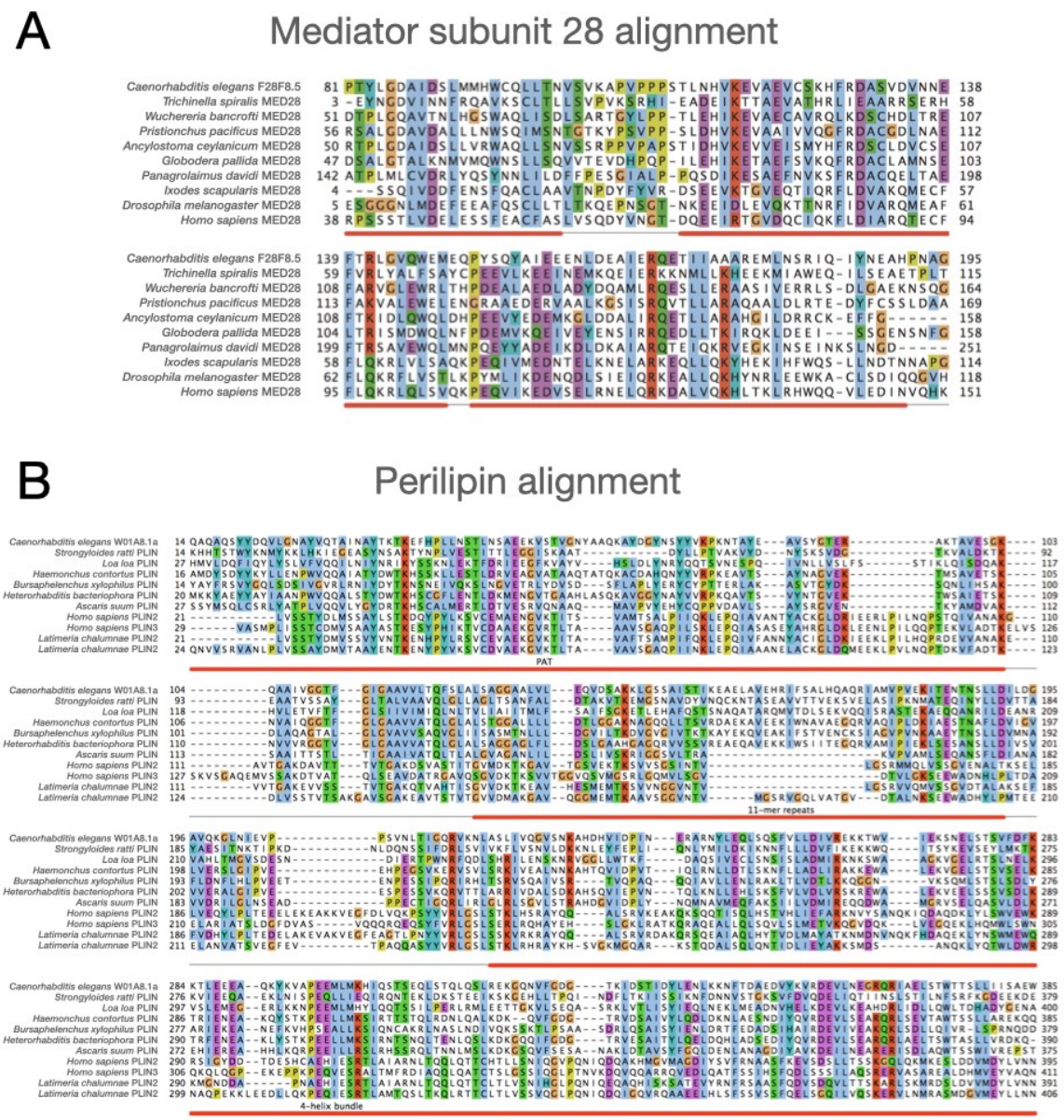


*Figure 23. Alignments of C. elegans protein sequences F28F8.5 and W01A8.1 with marked secondary structures. (A) Alignment of the amino-acid sequence of the conserved core section of F28F8.5 with Mediator subunit 28 homologues from representative organisms marked for simplicity everywhere MED28. (B) Alignment of amino-acid sequence of W01A8.1 with Perilipins of representative organisms. Red bars below alignments represent predicted α-helices and conserved tertiary structure motifs of PLINs are marked above the α-helices. Figure modified from (Kostrouchová et al., 2017) and (Chughtai et al., 2015) originally published under the Creative Commons Attribution and Public Domain Dedication Licences, respectively.*

We had been interested in the MED28 subunit due to its possible implications in negative regulation by otherwise positively regulating NRs. The absence of MED28 from *C. elegans* might have suggested that it is not indispensable for Mediator function including the negative regulation. Vladimir Saudek identified bioinformatically the transcript F28F8.5 as a potential orthologue for MED28 and the W01A8.1 as a potential orthologue for PLIN and he contacted us for experimental validation.

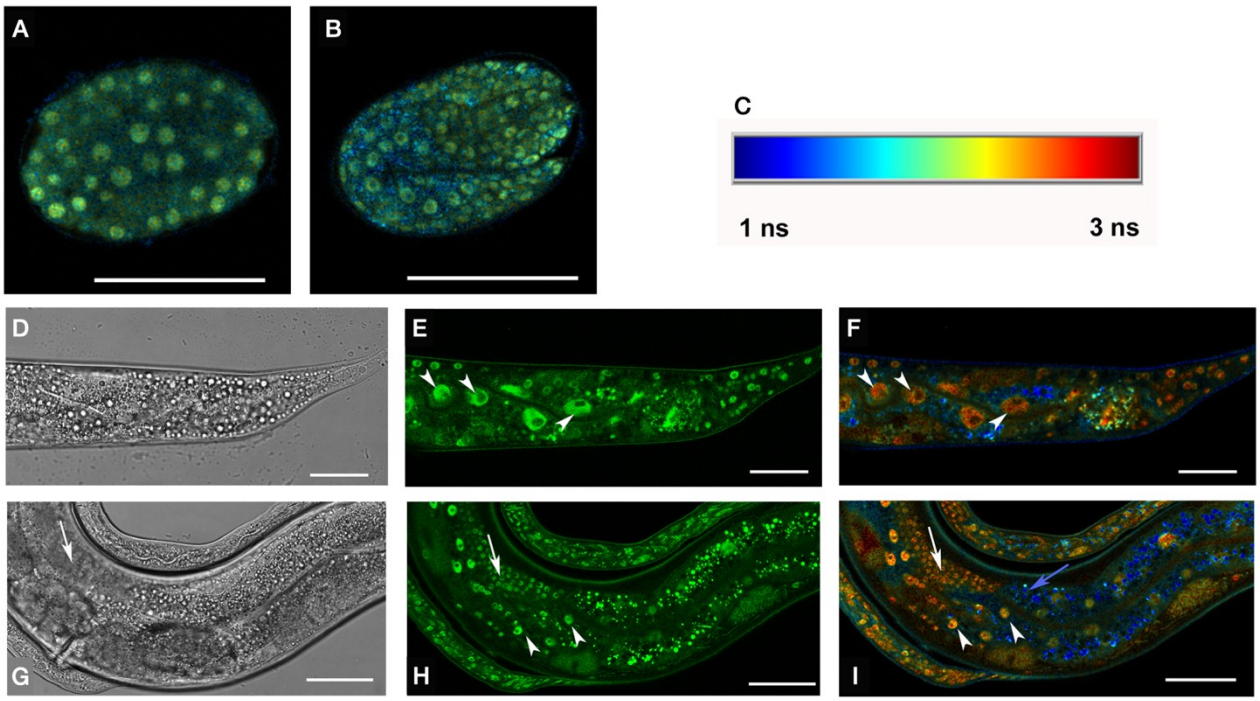


*Figure 24. Confocal and Fluorescence Lifetime Imaging Microscopy (FLIM) images of C. elegans embryos (A-B), larvae (on G-I) and adult hermaphrodite (D-I) expressing GFP::F28F8.5 from edited native gene. (A-B) FLIM diagrams of a bean stage (A) and a comma stage (B) embryos show nuclear localization of GFP signal with an average fluorescence lifetime (FL) of about 2 ns without significant autofluorescence at FL < 1.5 ns. (C) Applied calibration table for FL in the range 1 ns to 3 ns. (D and G) Nomarski optics brightfield micrographs of various parts of adult hermaphrodites and larvae corresponding to the images on their right. (E and H) Confocal fluorescent images of the same animals show fluorescence concentrated in nuclei, as well as in enterocyte granules. (F and I) FLIM diagrams of the same animals allow distinction between short lifetime fluorescence of the enterocyte granules (blue, average FL of 1 ns to 1.5 ns) and longer lifetime specific GFP fluorescence (green, yellow and red, average FL of 2.5 ns). Arrowheads point to enterocyte nuclei and arrows to germline nuclei. Blue arrow in (I) points germline turn. Bars indicate 30 μm in (A-B) and 50 μm in (D-I). Figure modified from (Kostrouchová et al., 2017) originally published under the Creative Commons Attribution Licence.*

We began by observing the subcellular localization of both proteins. Animals expressing GFP::F28F8.5 in place of the native F28F8.5 protein were imaged by fluorescent and confocal microscopy and FLIM to overcome interfering autofluorescence (presuming shorter lifetime of autofluorescence than that of the specific GFP fluorescence). In this way, F28F8.5 was found to be predominantly localized to nuclei, with only a smaller proportion of the protein localized to cytoplasm (Figure 24).

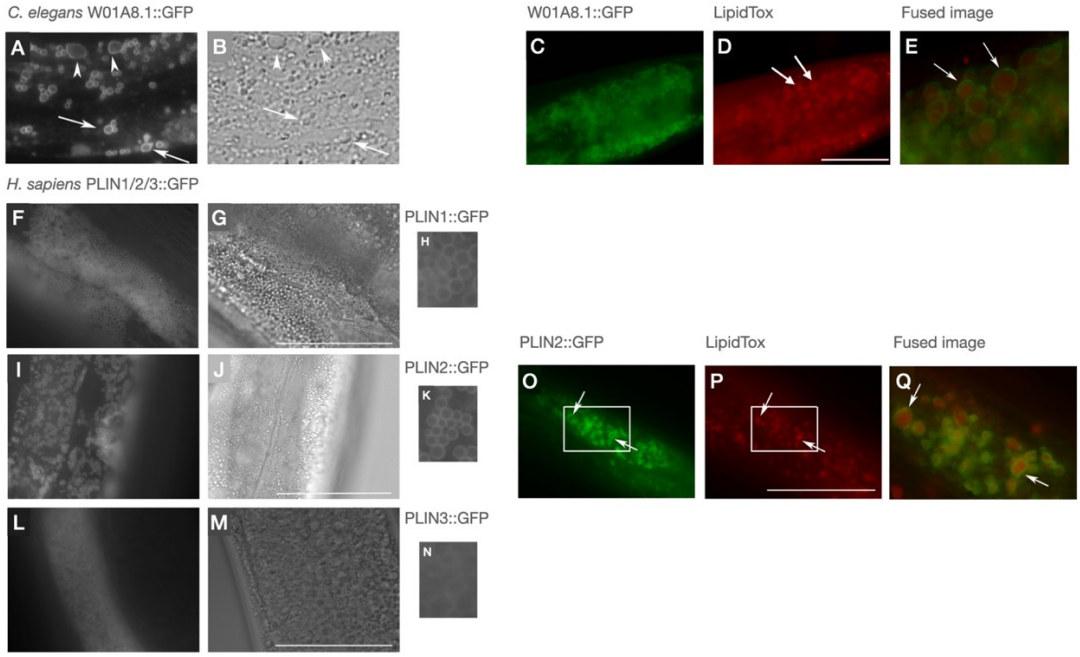


*Figure 25. Subcellular localization of C. elegans W01A8.1 (A-E) and human PLINs determined by fluorescent microscopy. (A) Fluorescent image of the middle body section of a young adult hermaphrodite expressing W01A8.1::GFP shows cytoplasmic concentration of the protein focalised around spherical structures in enterocytes and epidermal cells. (B) Corresponding brightfield microscopy image. (C) Green channel fluorescent image of W01A8.1::GFP expressing animal stained in red fluorescent LipidTox shows localization of W01A8.1. (D) Corresponding red channel image shows localization of neutral lipids in LDs. (E) Merged two-channel image of (C) and (D) attests the localization of W01A8.1 around LDs. (F) Fluorescent image of an animal expressing PLIN1::GFP show the same cytoplasmic localization of GFP signal. (G) Corresponding brightfield image. (H) A magnified crop-out of a fluorescent image of PLIN1::GFP animal showing spherical pattern. (I) Fluorescent image of an animal expressing PLIN2::GFP, its brightfield image (J) and a magnified crop-out (K). (L) Fluorescent image of an animal expressing PLIN3::GFP, its brightfield image (M) and a magnified crop-out (N). (O) Green channel fluorescent image of PLIN2::GFP expressing animal stained in red fluorescent LipidTox shows localization of human PLIN2 to the same structures as native W01A8.1. (P) Corresponding red channel image shows localization of neutral lipids in LDs. (Q) Merged two-channel image of (O) and (P) attests the localization of transgenic PLIN2 around LDs in C. elegans to the same extent as is the case of W01A8.1 (E). Bars represent 50 μm. Figure modified from (Chughtai et al., 2015) originally published under the Creative Commons Public Domain Dedication Licence.*

Synchronously, direct microscopic studies were performed on animals expressing W01A8.1::GFP prepared by injection of plasmid vectors, propagated in cells as extrachromosomal arrays. W01A8.1 was not present in the nucleus, as would be expected for a Mediator subunit, but rather in the cytoplasm. Specifically, the GFP signal was observed in enterocytes and epidermal cells concentrated around spherical vesicles. These vesicles were identified by LipidTox staining as lipid droplets (LDs) (Figure 25A-E). In the same way, transgene animals carrying human PLIN1, 2 and 3 transcripts fused with GFP, were prepared. All three PLIN paralogues localized to the same vesicles (LDs) as W01A8.1 (Figure 25F-Q).

A protein binding study of F28F8.5 was performed with nematode orthologues of the Mediator subunits 6 and 30 (MDT-6 and MDT-30), known to bind MED28 in other organisms. GST::F28F8.5 was incubated with either $His_6$::MDT-23::FLAG or $^{35}$S-Met-MDT-6 and the solution was resolved by polyacrylamide gel electrophoresis. The interaction documented by autoradiography or Western Blot confirmed specific binding of both MDT-6 and MDT-23 to F28F8.5 (Figure 26).

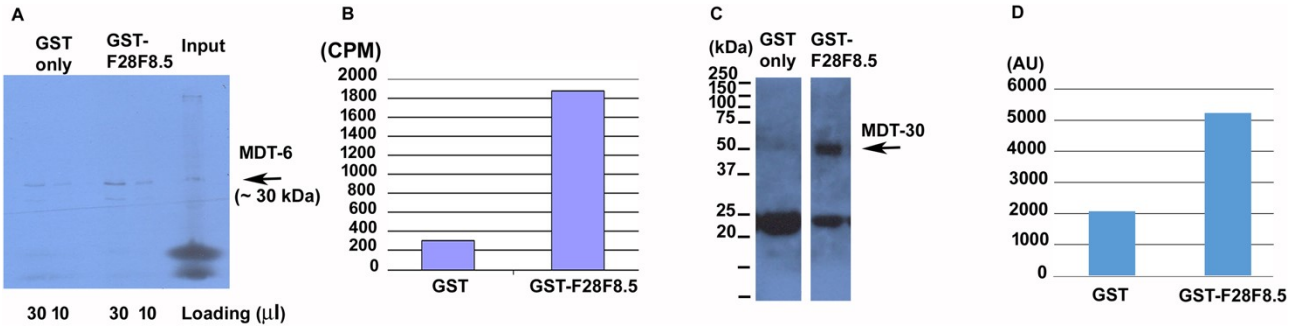


*Figure 26. Binding studies of F28F8.5 with MDT-6 and MDT-23. (A) Autoradiogram of SDS-PAGE separated GST control and GST::F28F8.5 after binding with radioactive $^{35}$S-Met-MDT-6 shows presence of a band corresponding to the MDT-6 protein in the GST::F28F8.5 column and only faint contamination band in the control experiment. (B) Quantification of radioactivity on cut-up dried gel defined as counts per minute (CPM) detected by a scintillation detector. (C) Western Blot analysis using anti-FLAG antibody of GST control and GST::F28F8.5 after binding with $His_6$::MDT-23::FLAG fusion protein shows a strong specific band at 50 kDa corresponding to the $His_6$::MDT-23::FLAG protein in the experiment and not in the GST control. (D) Quantification by digital densitometry of the Western Blot presented in (C) shows much higher density of the 50 kDa band in GST::F28F8.5 column defined by arbitrary units (AU). Figure modified from (Kostrouchová et al., 2017) originally published under the Creative Commons Attribution Licence.*

Having established homology of F28F8.5 with MED28 and the originally annotated *mdt-28* gene W01A8.1 with human PLINs, we followed by functional studies by RNAi-based down-regulation and gene knock-out. First, an injection RNAi protocol was applied to decrease somatic expression of

the F28F8.5 protein. Injection of dsRNA partially complementary to the *F28F8.5* gene sequence resulted in likely mosaic down-regulation of the *F28F8.5* gene expression with mixed phenotypes. 44% of the $F_1$ progeny of injected animals presented variously severe developmental defects, of which most notably embryonic and larval arrest, defective moulting, malformed vulvae, male tail ray defects, uncoordinated movements (Unc phenotype), embryo retention and vacuole formation, but the surviving animals were mostly fertile and proliferated (Figure 27A-D).

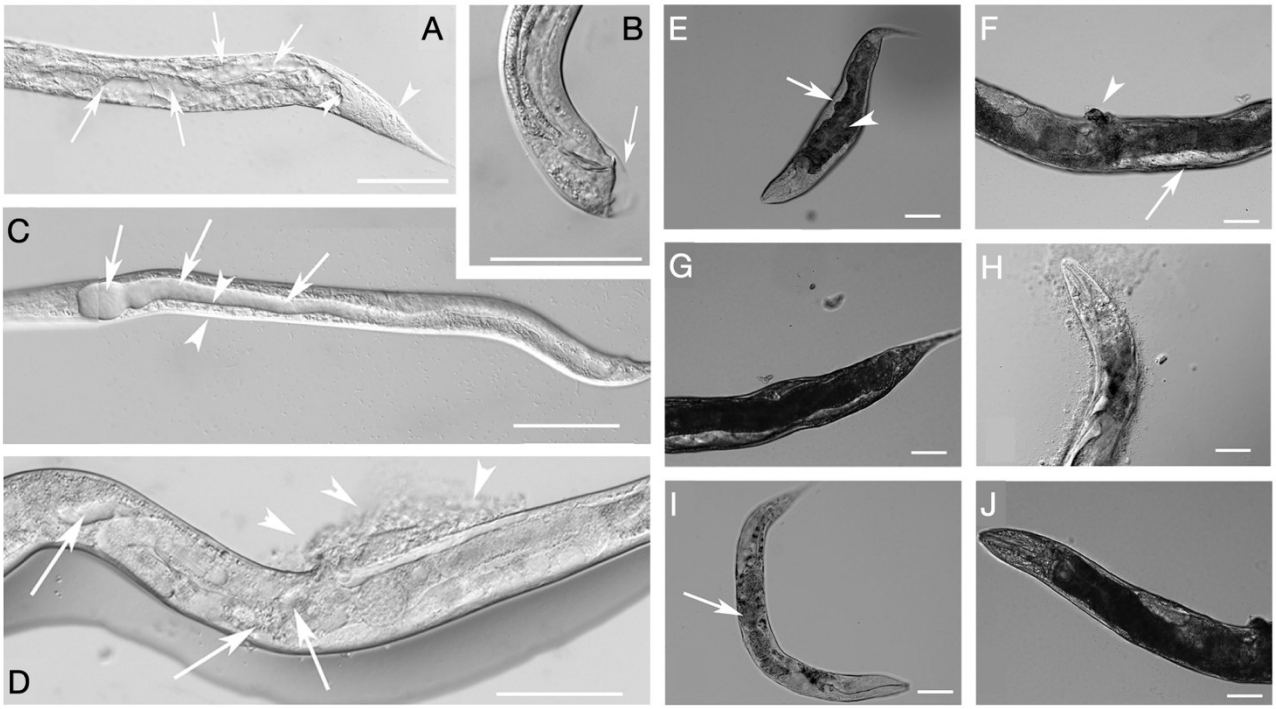


*Figure 27. Developmental defects of F28F8.5-defficient animals. (A)-(D) F28F8.5 expression down-regulation by RNAi. (E)-(J) F28F8.5$^{-/-}$ animals. Down-regulation phenotypes include: complete distortion of internal structure (A), male reproductive apparatus malformation (B), vacuole formation with gut atrophy (C), malformed vulva with explosion-through-vulva phenotype. Knock-out animals have unvaried dark intestine and distorted germlines, frequent dumpy phenotype (Dpy) (E), malformed vulvae (F), absent germline (G), malformed pharynges (H), distorted matrix in place of a germline (I) or a combination of the above-mentioned phenotypes (J). Bars represent 50 μm. Figure modified from (Kostrouchová et al., 2017) originally published under the Creative Commons Attribution Licence.*

We also prepared animals with a complete loss-of-function of the *F28F8.5* gene using CRISPR/Cas9-induced homologous recombination. A GFP-SEC sequence was inserted between the *F28F8.5* promoter and the gene itself, effectively completely hindering transcription of this gene. We found that homozygous disruption of the F28F8.5 reading frame was completely sterile and we propagated the line by selecting heterozygous animals expressing GFP alone in control of the *F28F8.5* promoter.

These animals had severe developmental deficits and structural deformations including: dumpy phenotype (Dpy), irregular darker gut, protruding vulvae (Pvul) and most prominently complete destruction of germline function and structure with the aforementioned complete sterility (Figure 27E-J). Expression analysis by qPCR confirmed a 17-fold decrease in F28F8.5 expression in these mutants, the residual mRNA likely originating in the maternal load of transcripts. Therefore, we observe that F28F8.5 is a vital protein for *C. elegans* that participates in tissue development.

Similarly, we studied the function of W01A8.1 by preparing *W01A8.1*$^{-/-}$ mutants via CRISPR/Cas9-induced gene deletion. Contrarily to F28F8.5 homozygous mutants, we observed that *W01A8.1*$^{-/-}$ animals experienced some embryonic malformations, defects in lipid metabolism regulation, but no lethality. *W01A8.1*$^{-/-}$ mutants were therefore thoroughly studied in different conditions in order to prove their implication in lipid metabolism rather than in gene expression regulation.

Structurally, *W01A8.1*$^{-/-}$ mutant adults have relatively conserved anatomy without apparent deformities, with completely preserved germline and almost unimpaired reproduction. However, we observe formation of large perinuclear vesicular structures in early embryos, disappearing around 6-cell stage. Using LipidTox staining and CARS imaging, we identify these structures as malformed enlarged LDs. CARS studies also show that adult tissues of *W01A8.1*$^{-/-}$ mutants contain conversely smaller LDs with lower total neutral lipid content (Figure 28).

In order to prove the implication of *W01A8.1* in lipid metabolism, we studied its functional connection to HOSL-1 by exposing animals to cold. In control animals with intact HOSL-1 lipolysis, only a small proportion of animal succumbed to the cold with a 93.07% surviving fraction and little structural deformities. As expected, *hosl-1*$^{-/-}$ mutants were extremely sensitive to cold exposure with only 25.21% survival. *W01A8.1*$^{-/-}$ mutants experienced mediocre cold tolerance with 43.99% surviving the experiment. Morphometric studies confirmed that *hosl-1*$^{-/-}$ mutants had 11-times and *W01A8.1*$^{-/-}$ mutants 6.5-times larger LDs than control animals, suggesting inefficient lipolysis in absence of these genes. Surpsrisingly, *W01A8.1*$^{-/-}$ *hosl-1*$^{-/-}$ double mutants had better survival and smaller LDs than *hosl-1*$^{-/-}$ single mutants, suggesting a presence of an alternative lipolytic pathway inhibited by W01A8.1 (Figure 29) (Kaššák et al., 2020).

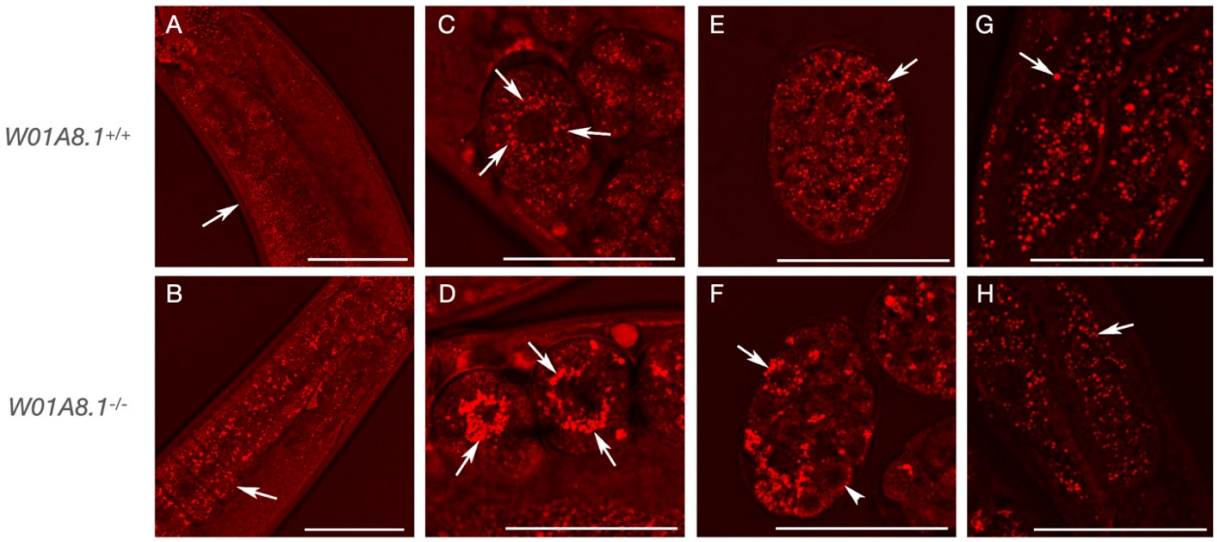


*Figure 28. Changes in LDs upon depletion of W01A8.1. CARS diagrams of controls (A), (C), (E) and (G) and of W01A8.1-defficient animals (B), (D), (F) and (H). (A) Germline of a control animal contains small regular LDs (arrows). (B) Germline of a mutant has enlarged LDs dispersed throughout the oocyte cytoplasm. (C) Early embryo of a control has small LDs concentrated around the nucleus. (D) Mutant embryos have distorted perinuclear neutral lipid accumulation. (E) Bean stage embryo of a control line shows regular finely dispersed LDs. (F) Late embryos of mutants have irregularly placed residual accumulations of distorted LDs. (G) Adult tissues of control animals have larger LDs with more neutral lipids than mutants (H). Bars represent 50 μm. Figure modified from (Chughtai et al., 2015) originally published under the Creative Commons Public Domain Dedication Licence.*

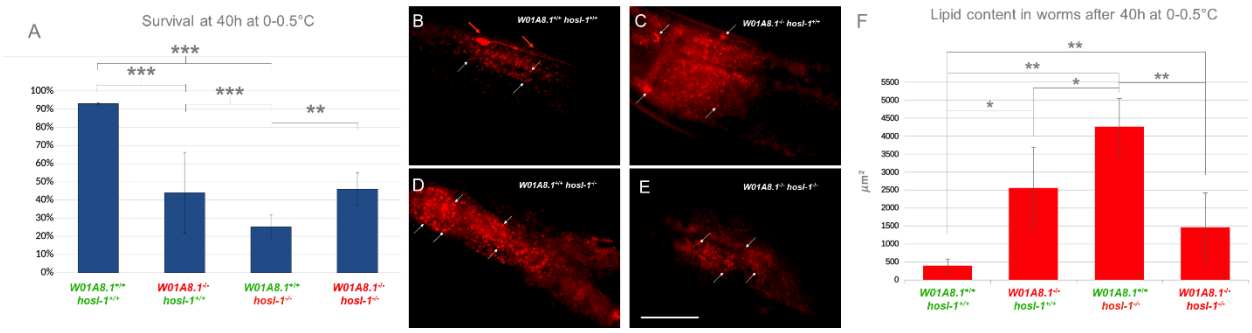


*Figure 29. Cold tolerance is affected by absence of HOSL-1 or W01A8.1. We followed survival fraction in wild-type controls, W01A8.1$^{-/-}$ mutants, hosl-1$^{-/-}$ mutants and W01A8.1$^{-/-}$ hosl-1$^{-/-}$ double mutants upon exposure to the temperatures of 0 °C to 0.5 °C during 40 h. (A) Surviving fractions of individual lines. (B)-(E) Representative red channel fluorescent microscopy pictures of LipidTox-stained surviving animals of controls (B), single mutants as marked (C) and (D) and double mutants (E). (F) Morphometric analysis of surviving LipidTox stained animals from a representative number of replicates. Bar represents 50 μm and images (B)-(E) are to scale. Significance description: * p < 0.05, ** p < 0.01, *** p < 0.001. Reused from (Kaššák et al., 2020) based on personal reuse by author agreement.*

In order to assess implication of autophagy in lipolysis, we crossed our *W01A8.1*$^{-/-}$ mutants with RD204 line expressing a GFP-bound autophagy marker LGG-1, under the control of a specific embryonal promoter $P_{pie-1}$. We imaged early embryos of the resulting $P_{pie-1}$-*lgg-1::gfp* x *W01A8.1*$^{-/-}$ line by green-channel confocal microscopy and by CARS. This way, we confirm a 3.1-fold increase ($p$ = 0.0069) in the volume of autophagic activity in early embryos in the absence of W01A8.1 associated with a 4.2-fold increase ($p$ = 0.041) in the total volume of LDs evidenced by CARS (Figure 30).

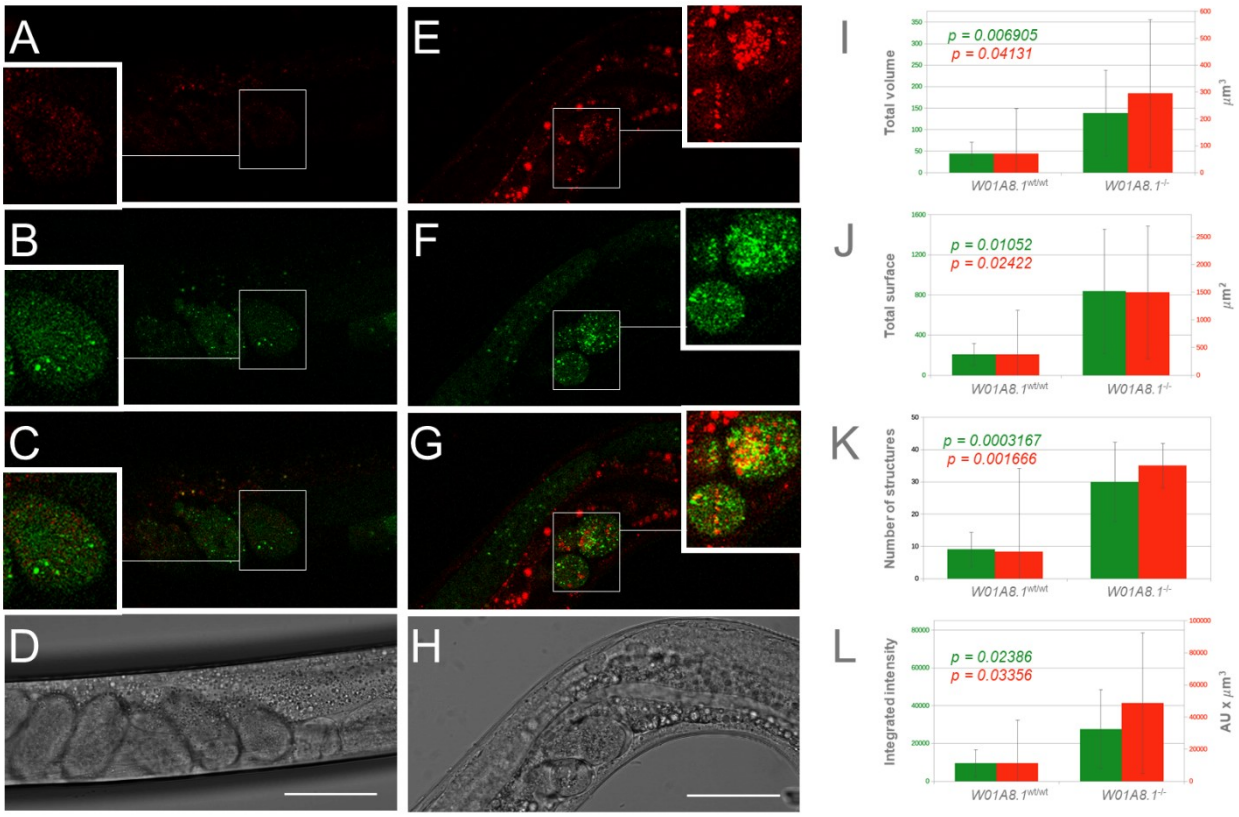


*Figure 30. Augmentation of LDs and activation of lipophagy upon W01A8.1 depletion. (A)-(D) Corresponding pictures of a representative animals of the control line RD204 [$P_{pie-1}$-lgg-1::gfp] with neutral lipids imaged by CARS (A), autophagy imaged by confocal microscopy of the LGG1::GFP in green channel (B). We present also merged CARS and green channel confocal image (C) and Nomarski optics brightfield image (D). (E)-(H) Corresponding CARS, green channel confocal, merged and Nomarski optics pictures of a representative animal of the KV2 line [$P_{pie-1}$-lgg-1::gfp x W01A8.1$^{-/-}$]. (I)-(L) Morphometric analyses of the GFP and CARS signal in a at least ten individual embryos. Defined as the total volume (I) of CARS or GFP positive structures, their total surface (J), their number (K) or their total integrated intensity (L). Bars indicate 50µm and images (A)-(H) are to scale. Cut-outs in images (A)-(C) and (E)-(G) are magnified and increased in brightness. Significance description: p values in image (first line: GFP, second line: CARS). Reused from (Kaššák et al., 2020) based on personal reuse by author agreement.*

# 4. Discussion

## 4.1. Evolutionary conservation of the entire NR/Mediator axis

In an evolutionary scope, the mechanisms of transcriptional regulation central for multicellular life developed gradually, as the structure of organisms increased in complexity. In intricate organisms such as ourselves, this functional determination of individual cells has reached inconceivable complexity and with it, certain fragility. The equilibria of regulations in our tissues permit differentiation and specialisation of individual cells that is subordinated to central regulation. Defects in this control result in carcinogenesis. However, even the most basic multicellular organisms require some tools for signal sensing and integration in order to maintain functional determination of individual cells. Throughout the animal kingdom, the most fundamental sensors and integrators of external and intercellular signals are the nuclear receptors (NRs) and their coregulators, of which most importantly the Mediator (Evans & Mangelsdorf, 2014; Rosenfeld et al., 2006).

Among the most conserved are the NRs principally responsible for metabolic regulations: RXRs and more recently TRs. TRs are the dominant metabolic regulators in vertebrates, where RXRs overtook a secondary function of dimerizing specificity filters and modulators of NR action. In non-chordate organisms of the subkingdom *Eumetazoa*, no TR homologues have been found and RXR is considered to play the primary role of a metabolic coordinator (Desvergne, 2007; Fonseca et al., 2020). Accordingly, RXR orthologues have been described in organisms as distant as *Cnidaria* (Kostrouch et al., 1998). The degree of conservation is so far-reaching that it could be questioned whether it did not appear in the genome of *Cnidaria* via a horizontal gene transfer from higher animals.

It this work, my colleagues and I discover and describe a functional orthologue of RXR in the most basal of multicellular animals, *Trichoplax adhaerens*. This confirms a direct evolutionary link between RXR homologues in *Cnidaria*, *Protostoma* and *Chordata*. Our discovery affirms the role of RXR as the fundamental NR at the base of the evolution of animal signalisation. We also observe that *in vivo* activation of TaRXR leads to the stimulation of the *L*-malate-$NADP^+$-oxidoreductase gene expression. This enzyme is considered one of the most important reporters of metabolic status and is specifically regulated by TR in vertebrates. This suggests the primordial role of RXR in metabolic regulation that was eventually partially overtaken by TRs.

It is believed that NRs in simple organisms developed to sense primarily external signals such as food gradients (Bertrand et al., 2004; Laudet, 1997). In our work, my colleagues and I confirm the connection between RXR signalling and food composition, but we find that this regulation is likely far more complex. In addition to RXR and a previously described ERR homologues, we confirmed the homologues of TNF-4 and of COUP-TF transcription factors in the *Trichoplax* genome. These four NRs seem to form a basic interconnected network. Upon activation of RXR by its ligand 9-*cis*-RA, the expression of RXR itself and of ERR is increased and that of COUP-TF is decreased. We postulate that this principle primes the simple endocrine network for differential sensing of food molecules, permitting the simplest forms of signal integration.

Elaborating on the subject of evolutionary conservation of the metabolic axis, we compared sequences from human RXRs, *T. adhaerens* RXR and human TRs. Although there is great functional homology throughout the LBDs of all these proteins, the homology of RXRs is far superior to that between human or *Trichoplax* RXRs and TRs (Figure 31). This suggests that TRs have developed independently from RXRs from one of the remaining NRs. This is in concord with evolutionary studies of TRs showing their closest NR paralogue being RAR (Wu, Niles, & LoVerde, 2007). Therefore, it is possible that metabolic regulation by RXR in *T. adhaerens* is also based on heterodimer formation with one of the remaining NRs. Unlike NRs, which are mostly restricted to the animal kingdom, the Mediator complex is conserved throughout the eukaryotic life. The core Head and Middle module subunits have conserved secondary structure in *Protista*, animals, plants and *fungi*. Some subunits are more restricted to individual kingdoms and higher animals have the most complex Mediator structure with 26 subunits excluding the CDK8-module. Inside the animal kingdom, the structure and function of individual subunits is again well conserved, in conformity with the conserved NR-based transcriptional regulation (Bourbon, 2008).

However, database searches identify MED28 as one of only two subunits whose *bona fide* orthologues could not have been identified with certainty throughout animal phyla by regular bioinformatical tools (Bourbon, 2008). In the crucial invertebrate model organism *Caenorhabditis elegans*, a gene in the locus W01A8.1 annotated as *mdt-28* had been considered the *MED28* orthologue (Punta et al., 2012). However, the little amount of previously available data rendered it the only Mediator subunit with seemingly unrelated structure and function in *C. elegans* and in mammals.

Importantly, W01A8.1 had not been shown to have nuclear localization and was actually found by proteomic studies to be one of the most abundant resident proteins on *C. elegans* lipid droplets (LDs)

(Zhang et al., 2012). Interestingly, *C. elegans* had been thought to lack an orthologue of LD-resident proteins Perilipins (PLINs) (J. H. Lee et al., 2014). These crucial proteins in lipid metabolism regulation are also extremely well conserved from human to invertebrates, *Protista* and *fungi*, but were largely considered to be absent from *C. elegans* genome. We postulated therefore that the *W01A8.1* gene, formerly annotated as *mdt-28*, was rather a Perilipin orthologue.

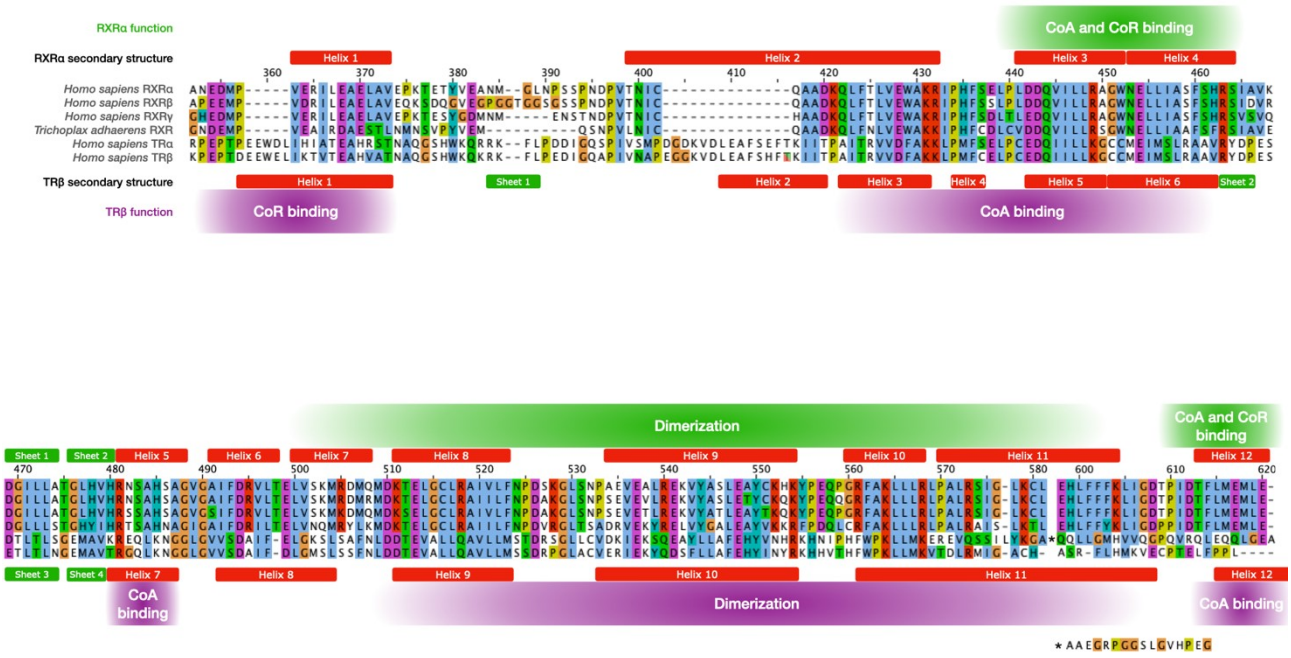


*Figure 31. Structural and functional alignment of RXR and TR LBDs shows considerable homology of these two NR lineages but no higher homology between TaRXR and TRs suggesting evolution of TRs from RXRs. Resolved secondary structure and functional domains of RXRs and TRs are shown above and below the alignment respectively. While sequences responsible for dimerization and coactivator binding are partially homologous, corepressor binding is catered for by N-terminal part of LBD in TRs and by AF2 subdomain in RXR. Two-turn sequence EEW(E/D)L seems crucial for this interaction. A green-red split highlight field of the T273 of TRβ sequence indicated the location of a mutation discovered in our work.*

Indeed, database searches, motif identifications and sequence alignments predicted with a high degree of probability that W01A8.1 is a *bona fide* orthologue of PLINs in *C. elegans* and that it is unrelated to MED28 (Figure 23B). Microscopic studies confirmed that W01A8.1 is a cytoplasmic protein without nuclear localisation and that it is concentrated around enterocyte and hypodermis vesicles identified by LipidTox staining as LDs (Figure 25A-E). Accordingly, human PLIN1, PLIN2 and

PLIN3 paralogues localise to identic vesicles in *C. elegans*, suggesting structural conservation (Figure 25F-Q) (Chughtai et al., 2015).

Next, an *ab initio* database search and alignments were performed to identify a *bona fide* orthologue of MED28 in *C. elegans*. An unannotated protein marked F28F8.5 was found that shared high core sequence homology with MED28 in other animals. By microscopic studies, we confirmed that unlike W01A8.1, F28F8.5 is predominantly nuclear protein, which aligns with its function as a Mediator subunit (Figure 24). We confirm the place of this protein in the Mediator complex by demonstrating its specific binding to two other Mediator subunit orthologues MDT-6 and MDT-30 (Figure 26), which are known to bind MED28 in human and other animals.

Based on our work, the annotation in WormBase database has been amended to identify W01A8.1 as *C. elegans* PLIN-1 (WormBase, 2015) and F28F8.5 as *C. elegans* MDT-28 (WormBase, 2017). Despite this, some authors continue to mistakenly refer to W01A8.1 as MDT-28/PLIN and to suggest functional homology between W01A8.1 and MED28 (Xie et al., 2019).

Therefore, we procured additional evidence in order to confirm the parallel homology of W01A8 and PLINs (Figures 28-31) and by it, of F28F8.5 and MED28 (Kaššák et al., 2020). Our functional studies of *W01A8.1*$^{-/-}$ mutants confirm that this protein intermediates activation of HOSL-1 (Figure 29) and inhibition of a secondary lipolytic mechanism, that we identify as lipophagy (Figure 30). These experiments therefore confirm the homology of W01A8.1 function with human PLINs (Figure 32) and provide strong evidence against its role as the annotated homologue of the Mediator subunit 28 (Kaššák et al., 2020).

Analysis of *F28F8.5* deficient nematodes found that they display a varied *tableau* of phenotypes due to differential maternal transcript load, but among constant traits are severe malformations of germline with no functional oogenesis and complete sterility (Figure 27). This suggests that F28F8.5 is important in proper tissue differentiation in *C. elegans*, as is the case of MED28 in human (*cf.* section 4.3.).

Put together, we established a high degree of conservation of the Mediator complex between human and *C. elegans* by identifying the correct conserved orthologue of an important subunit MED28 that was thought to be functionally not conserved in *C. elegans*. In this way, we completed and amended the current understanding in two distinct fields of *C. elegans* biology and strengthened the point of evolutionary conservation of the whole NR-Mediator axis.

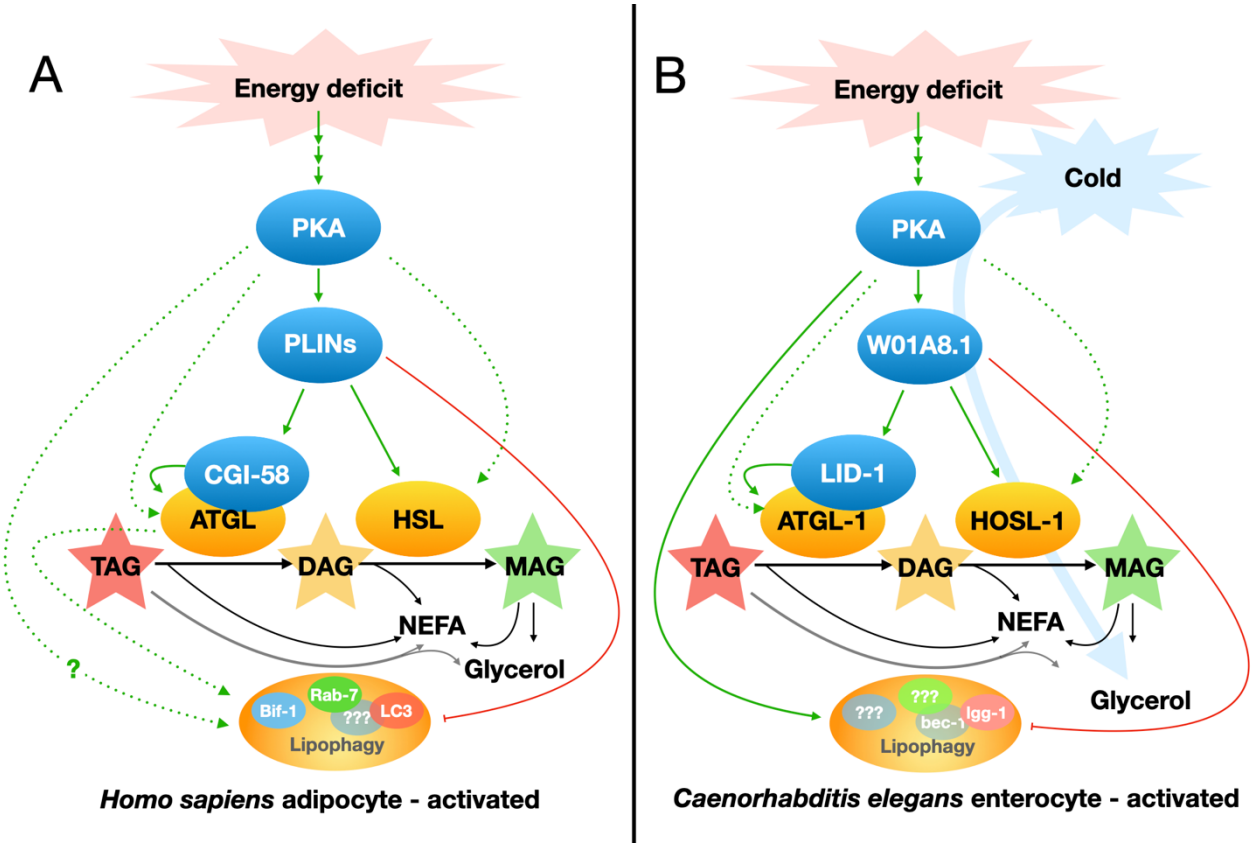


*Figure 32. Working model of lipolytic pathways in human and in C. elegans demonstrating functional conservation of human PLINs and W01A8.1. Need for energy liberation results in activation of the cAMP/Protein kinase A (PKA) signalling in both human and C. elegans. (A) Human PKA polyphosphorylates lipid droplet (LD)-resident PLINs, activating them. Phosphorylated PLINs activate hormone sensitive lipase (HSL) directly and adipocyte triglyceride lipase (ATGL) via its cofactor CGI-58. (B) Analogically in C. elegans, PKA activates W01A8.1 on LDs, which in turn activates ATGL-1 via its cofactor LID-1 and translocates HOSL-1 onto LDs (all respective orthologues of ATGL, CGI-58 and HSL). At the same time, W01A8.1 supresses lipophagy. In addition to its homologous function in energy liberation, the HOSL-1 dependent TAG degradation and glycerol liberation is also exploited in cold exposure in C. elegans.*

## 4.2. Negative regulation by TRβ

Having established the profound conservation of the metabolic regulation of gene expression by TR/RXR-Mediator axis, we may apply this model in molecular mechanical studies. As explained in section 1.4.3., NRs can regulate gene expression in the same cell both positively and negatively, *i.e.*, ligand binding to a NR can increase the expression of one gene and decrease the expression of another. The mechanisms behind this interesting principle are currently quite unclear.

A number of other sTFs regulate many genes negatively and the mechanisms are well established. What makes negative regulation by NRs cryptic is the way they bind their specific sequence of DNA.

The zinc finger coordinated DBD in all NRs is highly homologous and binds to identic or similar DNA motifs composed of six nucleotides called half-sites. The binding of NR monomers to half-sites is interchangeable, *i.e.* various NRs can bind to them. The specificity is assured via the organisation of two of these half-sites, making up a response element (RE). It could be presumed that positive and negative REs have different organisation of their individual half-sites permitting differential conformations of dimers and inverse signalling. This has been partially confirmed experimentally for RXR/TR heterodimer-based negative regulation (Chin et al., 1998). It was shown that not a single RE, but rather plurality of specific REs of positive and negative effect influences the expression of a single gene, suggesting signal integration at the level of a coregulator. The sequences of NR-binding half-sites of positive and negative REs do not seem to be exclusively based on the six nucleotides AGGT(C/A)A, but also to be strongly modulated by nucleotides immediately flanking these half-sites (Kim et al., 1992; Krishna et al., 1989). However, even these extended binding sequences cannot explain the full complexity of promoter determination of positively or negatively regulated genes (Nygård et al., 2006).

Apart from TRs, other NRs may also exhibit negative regulation on certain genes. In human, LXRα, PPARα, VDR and RARs have been shown to regulate negatively some genes (A. Li et al., 2019; Yan Liu et al., 2020; Strouhalova et al., 2020). All these NRs form heterodimers with RXRs. Indeed, RXR is seems to be required for the negative regulation by NR heterodimers (Chin et al., 1998; Laflamme et al., 2002). We searched for negative regulation in organisms with simplest regulation networks not possessing the complexity of specialised NRs. *T. adhaerens* only has four recognised NR genes, which is considered the minimum for regulation of gene expression in animals. We showed that while RXR and ERR expression is stimulated by the activation of RXR, COUP-TF is downregulated in the same conditions (Figure 21B). This suggests that even in the simplest organisms without subfamily 1 NRs, RXR alone might regulate certain genes negatively, likely in homodimers.

Primary and secondary structure of TRs and RXRs is conserved and most subdomains are responsible for the same functions. One notable exception is the AF2 module. In RXRs, AF2 structure is considerably simpler and dependent primarily on helix 12 with the aid of helices 3 and 4. The RXR AF2 is therefore less restrictive, binding both coactivators and corepressors (Figure 31).

As discussed in section 1.4.3., several mechanistical models can be envisaged for this negative regulation. Either liganded TR on a negative TRE (nTRE) interacts with a corepressor and unliganded TR on nTRE binds a coactivator, or the coregulator binding is not reversed, but rather the way these are bound causes allosteric changes in the coregulator complexes (Mediator or NCoR), which then

reverses the regulation. Structurally, it seems more plausible that in both positive and negative regulation, coactivators are bound via TR AF2 and RXR AF2 and corepressors via TR helix 1 and RXR AF2. In this model, RXR would change its position and conformation relative to the coregulatory complexes depending on whether it is bound to positive or negative REs, which is the premise of my work.

Indirect evidence suggests the validity of this presumption (Berghagen et al., 2002; Clifton-Bligh et al., 1998; Ortiga-Carvalho et al., 2005; Tagami et al., 1997; T. Takeda et al., 1997), but it is not universally accepted and it has yet to be verified structurally. However, structural studies by X-ray crystallography and nuclear magnetic resonance do not possess the capacity required to study these structures, such as the DNA-TR/RXR-Mediator-Pol II complexes. In fact, to date, no complete structures of the huge Mediator complex and the 270kDa NCoR proteins have been resolved. Modern methods of cryo-electron microscopy could eventually provide more information in this area, but these processes might be too dynamic to study statistically. Therefore, for the time being, we must rely on indirect functional studies.

As explained in sections 1.4.4. and 1.5.1., a convenient model to study TRβ function including the negative regulation is provided by the syndrome of resistance to thyroid hormone β (RTHβ). Patients suffering from this syndrome have more or less pronounced symptoms caused by dysregulation of the hypothalamus-pituitary-thyroid axis (Figure 11) due to dominant negative mutations in the *THRB* gene. Most of these mutations hamper the $T_3$-binding capacity causing complete functional deficit, but a minority interferes with other functions of the receptor, which can be used in reverse genetic studies.

Specifically, there is a minor group of mutations in TRβ inside the Cluster 3 (Figure 12) that affect the hinge domain and helix 1, impeding corepressor binding (Berghagen et al., 2002; Dumitrescu & Refetoff, 2013; Tagami et al., 1997). These patients have defective negative regulation by TRβ caused by an incapacity of the mutant receptors to bind corepressors and activate transcription of negatively regulated genes. Interestingly, NCoR2 (SMRT) has been shown to mediate also transcriptional activation in negatively regulated genes and this activation requires intact corepressor binding surface on TRs (Berghagen et al., 2002).

Another rarer group of mutations in *THRB* hinder the capacity of TRβ to bind coactivators via its AF2 surface (Dumitrescu & Refetoff, 2013). The mutation T273R newly discovered in our work belongs to this group. Patients in the family with T273R mutation suffer from a relatively milder form of

RTHβ with moderate biochemical phenotype of higher circulating $T_4$ and $T_3$ and high-normal to slightly elevated TSH levels, neuropsychological developmental issues and transitory infantile growth retardation and goitre. This clinical presentation is compatible with impaired positive signalling by TRβ causing hypothyroidism in some tissues and with impaired negative signalling causing non-suppression of TSH. However, the $T_3$ levels are only mildly increased and no hyperthyroidism symptoms are present, as is the case in mutations with impaired $T_3$-binding (Kaššák et al., 2017).

Architecturally, the threonine residue at the position 273 is localized in a N-terminal part of TRβ LBD helix 2. This structure and the T273 is completely conserved throughout vertebrate TRs and even in the simplest chordates, there is no larger or charged amino acid at this position (Figure 16). This is because the threonine residue at this position is packing tightly against the functionally crucial loop between helices 11 and 12. The first two turns of helix 2 are absent from RXRs, which can be explained by their simpler and functionally different structure of AF2 (Figure 31). While other mutations in the Cluster 3 neighbouring the T273R mutation have been known, these were mostly described in residues oriented towards the ligand binding pouch and are presumed to impair T3-binding or both T3 binding and coactivator binding (Dumitrescu & Refetoff, 2013).

In sum, both positive and negative regulation by TRβ is defective in patients with the T273R mutation and this is caused by a selectively impaired TRβ AF2 function. AF2 subdomain in TRs mediates binding of coactivators and not corepressors. Therefore, the discovery of this mutation finally confirms that coactivator binding by AF2 in $T_3$-bound TRβ mediates both transcription activation on positive TREs and transcriptional repression on negative TREs.

While these data corroborate the aforementioned model, what remains cryptic is the way in which coactivators revert their action to act as repressors and vice versa how corepressors activate gene expression. Importantly, one must consider that genes possess multiple positive and/or negative TREs and that likely multiple RXR/TR dimers bind to a coregulatory complex, which integrates the positive and negative signals and decides the sum effect on the expression of a single gene (Kim et al., 1992). In this sense, the role of the principal integrator, Mediator, can be presumed. The Mediator complex is considered principally as a coactivator, majority of its interactions leading ultimately to gene expression activation. However, as discussed in section 1.5.3., subunits 3 and 28 have been shown to inhibit gene expression upon the activation of Mediator (Beyer et al., 2007; Papamichos-Chronakis et al., 2000).

In our work, we corrected the data on MED28 conservation in *C. elegans* (Kostrouchová et al., 2017) and showed that this subunit along with the subunit MED1 are present and functionally homologous throughout *metazoa*, as far as NRs are utilised for gene expression regulation, but unrelated or absent from remaining evolutionary lineages. This correlation of prevalence might suggest functional connection. Most NRs, including TRs and RXRs, have been found to bind Mediator via the subunit 1 (Yuan et al., 1998). MED1 contains two coactivator consensus motifs LxxLL (NR boxes) that are bound by the NR AF2 surfaces. TRs, as well as other subfamily 1 proteins show preference towards NR box 2, leaving the NR box 1 available likely for RXR binding (Coulthard et al., 2003). Surprisingly, in *D. melanogaster,* recombinant RXR LBD with bound 9-*cis*-RA, but no other NRs, has been shown to bind another subunit, MED17, activating transcription of the reporter (Park et al., 2003). Once again, the situation is likely much more complex, seeing as some NRs interact with MED1 via different motifs (Jin et al., 2012; Wansa & Muscat, 2005) and in other cases, MED1 might be dispensable for transcriptional activation altogether (Ge et al., 2008). This only underlines the modularity of Mediator, making it the perfect intermediary for an inversed regulation.

In keeping with the vast body of current knowledge including our work on TRβ, RXR and MED28 on three various evolutionary levels, I elaborate on the currently proposed model of negative regulation by TRβ and propose a hypothetical mechanistic model. While it fits the presently available indirect data, it would require a direct verification, which might not be presently feasible due to the presented limitations of contemporary techniques. Coherently with the predominant current opinion, this model is based on inversed transcriptional activation by corepressors and inhibition by coactivators (Figure 33).

In $T_3$-unbound state, TR/RXR heterodimers are bound to NCoRs on both positive and negative TREs. Purposefully, NCoR1 but not NCoR2 (SMRT), contains the canonical coactivator LxxLL motif placed in between its RD1 and RD2 domains (Loinder & Söderström, 2004). NCoRs bind NRs usually via distinct RID1-3 domains and RXR AF2 binds preferentially LxxLL motif over the LxxxIxxxL motif. Therefore, we might speculate that on positive TREs, NCoR1 binds TR by one RID and RXR by the LxxLL motif, inhibiting transcription (Figure 33A), and on negative TREs, NCoR1/2 bind both TR and RXR by their RIDs or other parts of the NCoR molecule, activating transcription (Figure 33B).

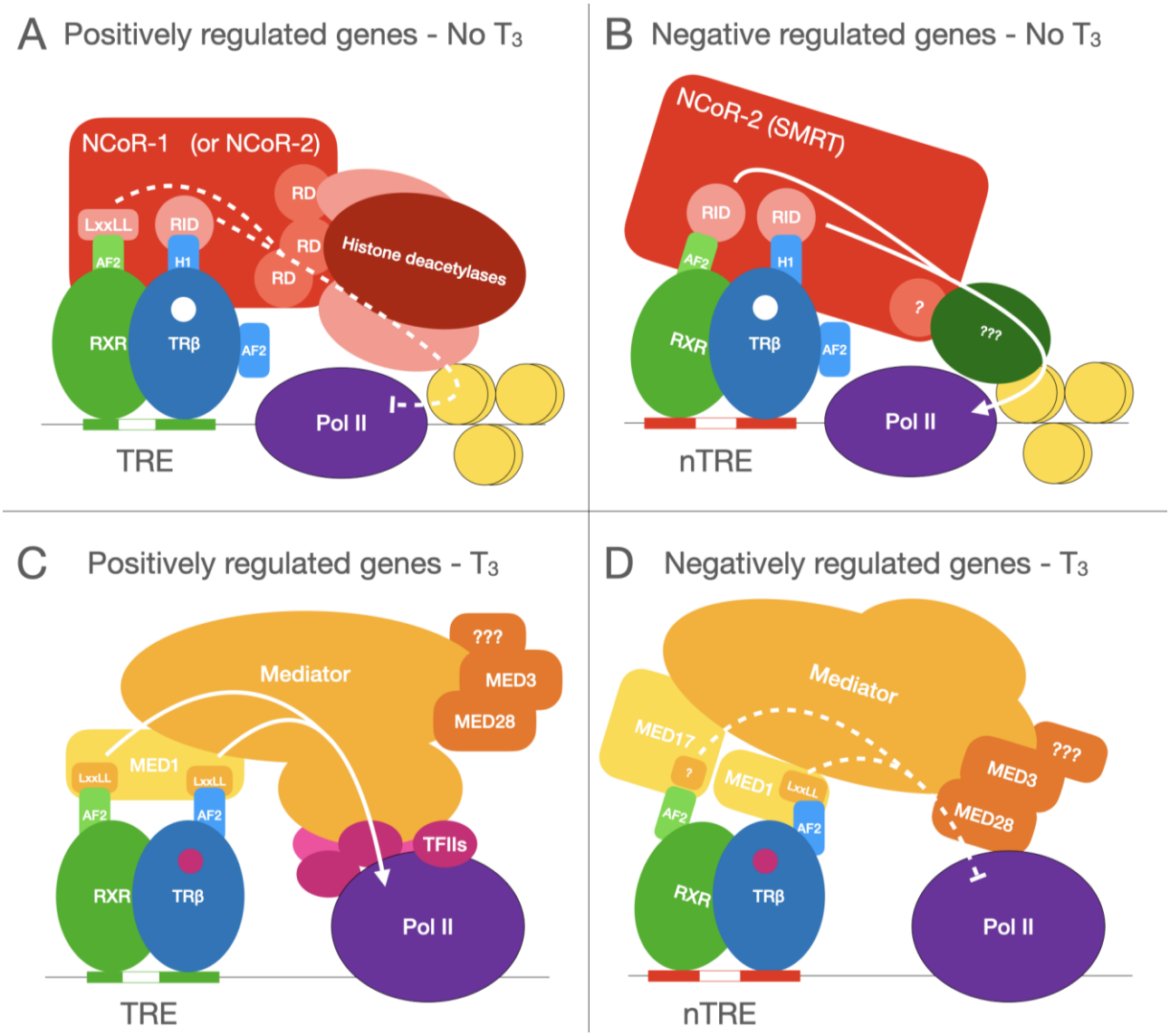


*Figure 33. A hypothetic model of transcription regulation by NRs on positive and negative TREs. In this concept, differential TRE organisation causes alteration in RXR/TRβ heterodimer conformation, ultimately changing the way coactivators and corepressors are bound by the dimer. In absence of the ligand, NCoRs are bound to RXR/TRβ dimers, regardless of the signalling direction, but differently. Positive TRE bound RXR/TRβ can interact with NCoR1 via one RID and one LxxLL motif that would be preferentially bound by RXRs (A). On negatively regulated genes, NCoR2 (SMRT) might be bound differently, e.g., via two RIDs, changing the conformation of NCoR2 and reverting the effect of NCoR2 on chromatin modifying complexes (B). When $T_3$ is bound to TRβ, NCoRs dissociate from its helix 1 (H1) and the AF2 subdomain changes conformation, actively binding the LxxLL motif on coactivators. It binds the NR box 2 on MED1 and RXR binds its NR box 1, activating Mediator and therefore gene expression (C). On negatively regulated genes, the different conformation of the RXR/TRβ dimer might cause differential binding of the Mediator complex, e.g., by the MED17 subunit, which in turn activates MED28 and/or MED3 which are known to mediate transcriptional silencing (D).*

This hypothetical model is the most basic possible mode of negative regulation of gene expression on specific genes. All the participating proteins and their functional domains are conserved throughout the animal phylogenetic tree down to *Placozoa* and possibly *Cnidaria*, as has been illustrated in our studies on *T. adhaerens* and *C. elegans*. Even at the basis of animal evolution in an organism only containing four NRs, negative regulation by NRs is likely present and important in a pseudo-endocrine signalling in metabolism and development. Therefore, a homologous model to the one described is likely to be valid throughout animal evolution, underlining its importance.

## 4.3. Implication in molecular oncology

In physiological conditions, NRs and Mediator are central regulators of gene expression, specifically in the metabolic and developmental context. As such, they are responsible for processing of intercellular signals leading to proliferation, differentiation, cell migration and apoptosis, translating these signals into a final effect of gene expression. All these functions belong to categories dysregulated in cancer, defined as hallmarks of cancer (Hanahan & Weinberg, 2000, 2011). It is therefore not surprising that NRs, as well as many of Mediator subunits were identified as pre-oncogenes and their dysregulation is an indispensable step in carcinogenesis (Dhiman et al., 2018; Weber & Garabedian, 2018).

Many parallels can be seen between ontogenesis in simple animals and cellular differentiation in complex organisms including human. It is this cellular differentiation and its control that are defective in malign neoplasms. Accordingly, among genes often dysregulated in cancer are TRs, RXRs and Mediator subunits implicated in development of invertebrates. My work has taken for aim to advance our understanding and provide novel capacities for study in these fields of fundamental research of these proteins, which could be ultimately applied in a clinical setting.

By describing a structurally and functionally conserved orthologue of RXR in *T. adhaerens*, we have equipped researchers in molecular oncology with a powerful new model system where RXR can be studied austere of the interaction noise of complex regulatory network, in an environment of only four NRs, yet applicable to human biology. I postulate that *T. adhaerens* could be used in preclinical development of specific agonist or antagonist ligands and in translational research of functional consequences of cancer-derived mutations in RXR genes.

Conversely, in the study of Mediator, we selected one of the best-established model organisms known to biology, *C. elegans*. We identified a discrepancy in structure and function of a subunit that caused

doubts in functional conservation of the Mediator complex. By correcting the previously erroneous annotation, we confirmed that the Mediator subunit 28 indeed has an orthologue in *C. elegans*, as does Perilipin. This opens a door for studies of the MDT-28, as the orthologue is now correctly annotated, and of the Mediator function in general. As introduced in section 1.5.3., MED28 is overexpressed in many cancers, driving carcinogenesis. As such, it is clearly a subject of interest in molecular oncological studies.

Furthermore, functional studies suggest that MED28 promotes oncogenicity via interreference and suppression of cellular differentiation programs. Specifically, defective MED28 signalisation has been implicated in the central event of epithelial-to-mesenchymal transition in carcinomas, in addition to other tumorigenic effects (M. F. Lee et al., 2011). These oncogenic properties have been associated with deregulated NRs (M. F. Lee et al., 2016) and depend on the negative regulation exerted by the MED28 subunit (Beyer et al., 2007). In this regard, the negative regulation by NRs is of utmost interest. Hence, we employed clinical translational research by reverse genetics in order to elucidate its enigmatic mechanism on the example of TRβ. Based on this and evolutionary studies of RXR (Figure 31), we propose a hypothetical mechanistic model of interactions between RXR, TRβ, MED1, MED17 and MED28 that might explain this regulation and could be exploited for therapeutic interference (Figure 33).

Highlighting the central position of NRs and Mediator in physiological and cancerous gene expression regulation, inhibition of MED28 and modulation of RAR function have been shown to decrease the expression of a whole array of oncogenes including cyclin D1, c-Myc, and nuclear β-catenin (Beyer et al., 2007). This underlines the importance of continuing molecular oncology studies of the NR-Mediator axis, in model organisms as well as in translational medical research (Brückmann et al., 2019; Porter et al., 2019; Schiano et al., 2014; Weber & Garabedian, 2018).

# 5. Conclusions

The multicellular life as we know it would not be possible without intercellular communication based on production, transmission, reception and integration of signals. In animals, among the most important receivers and integrators of signals are nuclear receptors (NRs) and their coregulators, most importantly the Mediator. The work connected to this thesis shed more light onto some of the most cryptic areas of gene expression regulation by NRs and the Mediator, while amending previous discrepancies in others.

Our work on RXR in *T. adhaerens* confirms a structural and functional conservation of NRs throughout the animal kingdom and elucidates how NRs adapted their function from external to intercellular sensors. Similarly, by amending and confirming the conservation of the MED28 orthologue in *C. elegans*, we reinforce the concept of a uniform structure and function of the whole Mediator complex throughout *Aminalia*.

This allows us to apply evolutionary studies of these proteins in our research of a particularly intriguing concept of negative regulation by NRs. Along with the reverse genetic studies mapping structural deficits against clinical phenotypes in a human syndrome of resistance to thyroid hormone β (RTHβ), these structural analyses allow recognition of crucial structural elements inside the TRβ, RXR and Mediator complex that participate on negative gene expression regulation.

Taken together, findings presented in this thesis contribute to the understanding of transcriptional regulation by NRs and Mediator and might have implications in cancer research.

# 6. References


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